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(54) Title: FOLATE MIMETICS AND FOLATE-RECEPTOR BINDING CONJUGATES THEREOF

(57) Abstract: A cell population expressing folate receptors is selectively targeted with a folate mimetic. The folate mimetic is conjugated to a diagnostic or therapeutic agent to enable selective delivery of the agent to the targeted cell population.

5 FOLATE MIMETICS  
AND FOLATE-RECEPTOR BINDING CONJUGATES THEREOF

This application claims the benefit of U.S. Provisional Application Serial No. 60/286,082, filed April 24, 2001, which is incorporated herein by reference in its entirety.

10

*Statement of Government Rights*

This invention was made with Government support under Grant R01-CA70845 awarded by the National Institutes of Health - National Cancer Institute. The Government has certain rights in the invention.

15

*Field of the Invention*

This invention relates to folate mimetics and their use in therapeutic and diagnostic applications. More particularly, this invention relates to using des-glutamyl folic acid analogs recognized by and selectively bound by folate  
20 receptors and other folate binding proteins and the use of such analogs for targeted delivery of diagnostic or therapeutic agents to folate-receptor bearing cell populations.

*Background of the Invention*

25 A number of methods are known for selectively targeting cells in a patient for delivery of diagnostic or therapeutic agents. Selective targeting has led to the introduction of various diagnostic agents for visualization of tissues, such as contrast agents useful in Magnetic Resonance Imaging (MRI), radiodiagnostic compositions, and the like. Introduction of therapeutic agents,  
30 such as compositions for radiotherapy or for neutron capture therapy, compositions for chemotherapy, various proteins, peptides, and nucleic acids, protein toxins, antisense oligonucleotides, liposomes, analgesics, antibiotics, antihypertensive agents, antiviral agents, antihistamines, expectorants, vitamins, plasmids, and the like, has also been demonstrated.

- Folate conjugates have been used for the selective targeting of cell populations expressing folate receptors or other folate binding proteins to label or deliver bioactive compounds to such cells. The relative populations of these receptors and binding proteins have been exploited in achieving selectivity in the targeting of certain cells and tissues, such as the selective targeting of tumors expressing elevated levels of high-affinity folate receptors. The following publications, the disclosures of which are incorporated herein by reference, illustrate the nature and use of folate conjugates for diagnosis or delivery of biologically significant compounds to selected cell populations in patients in need of such diagnosis or treatment:
- (a) Leamon and Low, "Cytotoxicity of Momordin-folate Conjugates in Cultured Human Cells" in *J. Biol. Chem.*, 1992, 267, 24966-24967.
  - (b) Leamon *et al.*, "Cytotoxicity of Folate-pseudomonas Exotoxin Conjugates Towards Tumor Cells" in *J. Biol. Chem.*, 1993, 268, 24847-24854.
  - (c) Lee and Low, "Delivery of Liposomes into Cultured Kb Cells via Folate Receptor-mediated Endocytosis" in *J. Biol. Chem.*, 1994, 269, 3198-3204.
  - (d) Wang *et al.*, "Delivery of Antisense Oligonucleotides Against the Human Epidermal Growth Factor Receptor into Cultured Kb Cells with Liposomes Conjugated to Folate via Polyethyleneglycol" in *Proc. Natl. Acad. Sci. U.S.A.*, 1995, 92, 3318-3322.
  - (e) Wang *et al.*, "Synthesis, Purification and Tumor Cell Uptake of Ga-67-deferoxamine-folate, a Potential Radiopharmaceutical for Tumor Imaging" in *Bioconj. Chem.*, 1996, 7, 56-63.
  - (f) Leamon *et al.*, "Delivery of Macromolecules into Living Cells: a Method That Exploits Folate Receptor Endocytosis" in *Proc. Natl. Acad. Sci., U.S.A.*, 1991, 88, 5572-5576.
  - (g) Krantz *et al.*, "Conjugates of Folate Anti-Effector Cell Antibodies" in U.S. Patent No. 5,547,668.

- (h) Wedeking *et al.*, "Metal Complexes Derivatized with Folate for Use in Diagnostic and Therapeutic Applications" in U.S. Patent No. 6,093,382.
- (i) Low *et al.*, "Method for Enhancing Transmembrane Transport of Exogenous Molecules" in U.S. Patent No. 5,416,016.
- 5 (j) Miotti *et al.*, "Characterization of Human Ovarian Carcinoma-Associated Antigens Defined by Novel Monoclonal Antibodies with Tumor -Restricted Specificity" in *Int. J. Cancer*, 1987, 39, 297-303.
- (k) Campell *et al.*, "Folate-Binding Protein is a Marker for Ovarian Cancer" in *Cancer Res.*, 1991, 51, 5329-5338.
- 10 (l) Jansen *et al.*, "Identification of a Membrane-Associated Folate-Binding Protein in Human Leukemic CCRF-CEM Cells with Transport-Related Methotrexate Resistance" in *Cancer Res.*, 1989, 49, 2455-2459.

Multiple types of folate recognition sites present on cells, such as  $\alpha$ -folate receptors,  $\beta$ -folate receptors, folate binding proteins, and the like, have  
15 been shown to recognize and bind the conjugates described above. The primary pathway for entry of folate derivatives into cells is through a facilitated transport mechanism mediated by a membrane transport protein. However, when folate is covalently conjugated to certain small molecules and macromolecules, the transport system can fail to recognize the folate molecule.

20 Advantageously, in addition to the facilitated transport protein, some cells possess a second membrane-bound receptor, folate binding protein (FBP), that allows folate uptake via receptor-mediated endocytosis. At physiological plasma concentrations (nanomolar range), folic acid binds to cell surface receptors and is internalized via an endocytic process. Receptor-mediated  
25 endocytosis is the movement of extracellular ligands bound to cell surface receptors into the interior of the cells through invagination of the membrane, a process that is initiated by the binding of a ligand to its specific receptor. The uptake of substances by receptor-mediated endocytosis is a characteristic ability of some normal, healthy cells such as macrophages, hepatocytes, fibroblasts,  
30 reticulocytes, and the like, as well as abnormal or pathogenic cells, such as tumor cells. Notably, folate binding proteins involved in endocytosis are less

sensitive to modification of the folate molecule than the membrane transport proteins, and often recognize folate conjugates. Both targeting and uptake of conjugated diagnostic and therapeutic agents are enhanced.

Following endosome acidification, the folate receptor changes  
5 conformation near its ligand-binding domain and releases the folic acid molecule. Folate receptors are known to recycle back to the membrane surface for additional rounds of ligand-mediated internalization. However, a significant fraction of the internalized receptor-folic acid complex has been shown to return back to the cell surface shortly after endocytosis. This suggests that the  
10 acid-triggered ligand release mechanism does not proceed to completion, at least after the first round of internalization (Kamen et al., 1988, *J. Biol. Chem.* 263, 13602-13609).

Pteric acid, which is essentially folic acid lacking the distal glutamyl residue (Figure 1), does not bind to the high-affinity folate receptor to any  
15 appreciable extent (Kamen et al., 1986, *Proc. Natl. Acad. Sci., U.S.A.* 83, 5983-5987); in fact, 2  $\mu$ M pteric acid (100-fold excess) had absolutely no effect on the binding of folate to the folate receptor. Thus, the glutamyl residue of folic acid, or some portion thereof, was generally thought to be required for efficient, specific receptor recognition. However, recent studies have revealed that the  
20 glutamyl residue of folic acid could be replaced with a lysyl residue without disturbing the binding affinity of the ligand (McAlinden et al., 1991, *Biochemistry* 30, 5674-5681.; Wu et al., 1997, *J. Membrane Biol.* 159, 137-147), that the glutamyl residue can be replaced with a glycyl residue without substantially altering cellular uptake, and that no selective isomeric (i.e.,  $\alpha$ -  
25 glutamyl vs.  $\gamma$ -glutamyl) conjugation requirement necessarily exists (Leamon et al., *J. Drug Targeting* 7:157-169 (1999); Linder et al., *J. Nuclear Med.* 41(5):470 Suppl. 2000).

Efforts to improve the selectivity of targeting or increase the diversity of the agents delivered to the cell or tissue have been hampered by a number of  
30 complications, including the complex syntheses required for the preparation of these conjugates. Such synthetic schemes are not only time consuming, but

may also preclude the use of certain conjugates due to synthetic incompatibilities. A folic acid analog capable of expanding the number or diversity of agents, via the conjugates of such agents and these folic acid analogs, presentable to target cells would be advantageous.

5

### *Summary of the Invention*

The present invention provides a compound that is capable of binding as a ligand to a folate recognition site. The compound is referred to as a "non-peptide folic acid analog."

10 The present invention also provides a ligand-agent conjugate capable of binding to a folate recognition site, the ligand-agent conjugate comprising a diagnostic or therapeutic agent in association with a non-peptide folic acid analog.

15 The present invention also provides a ligand-agent conjugate capable of binding to a folate recognition site with high affinity, the ligand-agent conjugate comprising a diagnostic or therapeutic agent in association with a plurality of non-peptide folic acid analogs.

20 The present invention also provides a method for targeting a cell or tissue with a diagnostic or therapeutic agent, comprising the step of administering to a patient an effective amount of a ligand-agent conjugate comprising a diagnostic or therapeutic agent in association with a non-peptide folic acid analog.

### *Brief Description of the Drawings*

25

Figure 1 is a schematic representation of folic acid and pteronic acid.

Figure 2 is a schematic representation of the synthesis of a pteronic acid conjugate, CYK4-013.

30 Figure 3 is a schematic representation of the synthesis of pteronic acid conjugate linked to the tetraazamacrocyclic DOTA chelating ligand (CY4-036).

Figure 4 is a schematic representation of the metabolism of a ligand-agent conjugate of the invention involving bioreduction to release the agent.

Figure 5 is a schematic representation of the metabolism of a ligand-agent conjugate of the invention involving acid hydrolysis to release the agent.

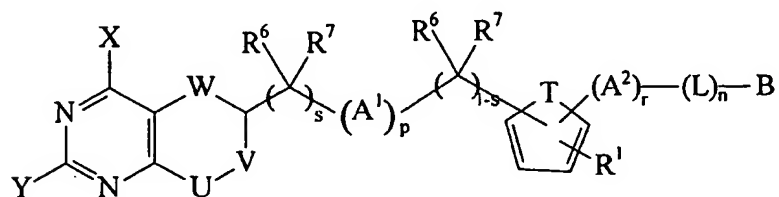
5 Figure 6 depicts the binding activity of (S)- $\alpha$ -carboxybenzoyl pteroate (ACBP) and N-pteroyl-2-amino-2-carboxymethylpyridine (Pte-AP).

Figure 7 is a schematic representation of the synthesis of pteroylhydrazido-benzenetetracarboxylic acid-diacetoxyscirpenol (Pte-hydrazideo-BTCA-DAS).

10 Figure 8 depicts the binding activity of pteroyl hydrazide (Pte-hydrazide) and Pte-hydrazido-BTCA-DAS.

#### *Detailed Description of the Preferred Embodiments*

15 The present invention provides a ligand capable of binding to a folate recognition site, comprising a non-peptide folic acid analog of general formula I:



I

20 wherein

X and Y are each independently selected from the group consisting of halo,  $R^2$ ,  $OR^2$ ,  $SR^3$ , and  $NR^4R^5$ ;

U, V, and W represent divalent moieties each independently selected from the group consisting of  $-(R^6)C=$ ,  $-N=$ ,  $-(R^6)C(R^7)-$ , and  $-N(R^4)-$ ;

T is selected from the group consisting of S, O, N and  $-C=C-$  such that the ring structure of which T is a member is aromatic;

$A^1$  and  $A^2$  are each independently selected from the group consisting of  $-C(Z)-$ ,  $-C(Z)O-$ ,  $-OC(Z)-$ ,  $-N(R^{4'})-$ ,  $-C(Z)-N(R^{4'})-$ ,  $-N(R^{4'})-C(Z)-$ ,  
 5  $-O-C(Z)-N(R^{4'})-$ ,  $-N(R^{4'})-C(Z)-O-$ ,  $-N(R^{4'})-C(Z)-N(R^{5'})-$ ,  $-O-$ ,  $-S-$ ,  
 $-S(O)-$ ,  $-S(O)_2-$ ,  $-N(R^{4'})S(O)_2-$ ,  $-C(R^{6'}) (R^{7'})-$ ,  $-N(C\equiv CH)-$ ,  
 $-N(CH_2-C\equiv CH)-$ ,  $C_1-C_{12}$  alkyl and  $C_1-C_{12}$  alkoxy; where Z is oxygen or sulfur;

$R^1$  is selected from the group consisting of hydrogen, halo,  $C_1-C_{12}$  alkyl,  
 10 and  $C_1-C_{12}$  alkoxy;

$R^2$ ,  $R^3$ ,  $R^4$ ,  $R^{4'}$ ,  $R^5$ ,  $R^{5'}$ ,  $R^{6'}$  and  $R^{7'}$  are each independently selected from the group consisting of hydrogen, halo,  $C_1-C_{12}$  alkyl,  $C_1-C_{12}$  alkoxy,  $C_1-C_{12}$  alkanoyl,  $C_1-C_{12}$  alkenyl,  $C_1-C_{12}$  alkynyl,  $(C_1-C_{12}$  alkoxy)carbonyl, and  $(C_1-C_{12}$  alkylamino)carbonyl;

15  $R^6$  and  $R^7$  are each independently selected from the group consisting of hydrogen, halo,  $C_1-C_{12}$  alkyl, and  $C_1-C_{12}$  alkoxy; or,  $R^6$  and  $R^7$  are taken together to form  $O=$ ;

$R^{6'}$  and  $R^{7'}$  are each independently selected from the group consisting of hydrogen, halo,  $C_1-C_{12}$  alkyl, and  $C_1-C_{12}$  alkoxy; or,  $R^{6'}$  and  $R^{7'}$  are taken  
 20 together to form  $O=$ ;

L is a divalent linker;

n, p, r and s are each independently either 0 or 1; and

B is hydrogen or a leaving group;

provided that the linker L does not include a naturally occurring amino  
 25 acid covalently linked to  $A^2$  at its  $\alpha$ -amino group through an amide bond. It should be understood that the structure of formula I includes tautomeric structures, for example in compounds where X is OH, SH or NH.

In the compound of the invention wherein any one or more of  $A^1$ ,  $A^2$ ,  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$ ,  $R^{4'}$ ,  $R^5$ ,  $R^{5'}$ ,  $R^{6'}$ ,  $R^{7'}$ ,  $R^6$ ,  $R^7$ ,  $R^{6'}$  and  $R^{7'}$  comprises an alkyl,  
 30 alkoxy, alkylamino, alkanoyl, alkenyl, alkynyl, alkoxy carbonyl, or alkylamino



carbonyl group, the group preferably contains 1 to 6 carbon atoms ( $C_1-C_6$ ); more preferably it contains 1 to 4 carbon atoms ( $C_1-C_4$ ).

Folic acid contains a glutamyl residue bound at its  $\alpha$ -amino group via an amide bond to the benzoate moiety of pteric acid (Figure 1). This amide bond  
5 would typically not be classified as a "peptide" bond because pteric acid is not an amino acid; a peptide bond is typically characterized as a bond in which the carboxyl group of one amino acid is condensed with the amino group of another to form a – CO.NH- linkage.

Nonetheless, for ease of reference, the compound of formula I, which is  
10 defined as having linker L that lacks a glutamyl or any other naturally occurring amino acid residue covalently linked to  $A^2$  at its  $\alpha$ -amino group through an amide bond, is termed herein a "non-peptide" folic acid analog. That is, the term "non-peptide" as used herein in reference to the compound of formula I, means that linker L does not include a naturally occurring amino acid  
15 covalently linked to  $A^2$  through an amide bond at its  $\alpha$ -amino group, thereby distinguishing the compound of formula I from, for example, folic acid, pteroyl- $\gamma$ -glutamate-cysteine, pteroyl- $\alpha$ -glutamate-cysteine, and pteroyl-glycine-cysteine (Leamon et al., *J. Drug Targeting* 7:157-169 (1999)). In a preferred embodiment of the non-peptide folic acid analog of the invention,  
20 linker L does not include any amino acid (whether naturally occurring or non-naturally occurring) covalently linked to  $A^2$  through an amide bond at its  $\alpha$ -amino group.

It should be further understood that the compound of formula I can contain a naturally occurring amino acid covalently linked to  $A^2$  at a site other  
25 than its  $\alpha$ -amino group, a non-naturally occurring amino acid covalently linked to  $A^2$  through an amide bond or otherwise, as well as any other non-amino acid moiety covalently linked to  $A^2$  through an amide bond or otherwise, as defined with reference to the formula.

The general chemical terms used in the formulae above have their usual  
30 meanings. For example, the term "alkyl" as used herein refers to a linear or branched chain of carbon atoms, such as methyl, ethyl, *n*-propyl, isopropyl, *n*-

butyl, isobutyl, *sec*-butyl, *tert*-butyl, pentyl, 2-pentyl, 3-pentyl, neopentyl, hexyl, heptyl, octyl and the like.

The term "alkoxy" as used herein refers to alkyl, as defined above, substituted with oxygen, such as methoxy, ethoxy, propoxy, isopropoxy, butoxy, *tert*-butoxy and the like.

The term "alkanoyl" as used herein refers to formyl, or alkyl, as defined above, terminally-substituted with a carbonyl such as acetyl, propanoyl, butanoyl, pentanoyl and the like.

The term "alkenyl" as used herein refers to a linear or branched chain of carbon atoms with one or more carbon-carbon double bonds, such as vinyl.

The term "alkynyl" as used herein refers to a linear or branched chain of carbon atoms with one or more carbon-carbon triple bonds.

The term "alkylamino" as used herein refers to alkyl, as defined above, substituted with nitrogen, including both monoalkylamino such as methylamino, ethylamino, propylamino, *tert*-butylamino, and the like, and dialkylamino such as dimethylamino, diethylamino, methylpropylamino, and the like.

The term "halo" as used herein refers to any Group 17 element and includes fluoro, chloro, bromo, iodo, and astatine(o).

The term "alkylenyl" as used herein refers to a divalent linear or branched chain of carbon atoms such as methylene, ethylene, 2-methylpropylene, and the like.

The term "leaving group" as used herein refers to a functionality that may be replaced, such as an activated halo or alkoxy, by an introduced substituent, such as a alkylamino, carbon nucleophile, a different alkoxy, a different halo, and the like.

The term "naturally occurring amino acid" as used herein refers to the 20 coded amino acids available for endogenous protein synthesis, such as glycine, alanine, methionine, and the like.

A preferred embodiment of the ligand is one having the general formula I wherein p is 1, s is 1, and T, U, V, W, X, Y, R<sup>1</sup>, R<sup>6</sup>, R<sup>7</sup> and A<sup>1</sup> are selected

such that at least a portion of the molecule is isosteric with pterioic acid. By "isosteric" it is meant that the two compounds or portions of compounds comprise isosteric substituents that occupy similar volumes and, preferably but not necessarily, have similar electronic character. As a nonlimiting example,  
 5 hydrogen, halo, CH<sub>3</sub>, OH, SH and NH<sub>2</sub> may be considered for purposes of this invention as being isosteric substituents.

Folate receptor activity is expected to be retained when isosteric substitutions are made to that portion of the non-peptide folic acid analog that is derived from pterioic acid. For example, as reported in Jansen, "Receptor- and  
 10 Carrier-Mediated Transport Systems for Folates and Antifolates," in *Anticancer Drug Development Guide: Antifolate Drugs in Cancer Therapy*, Jackman, Ed., Humana Press Inc, Totowa NJ (1999), ring substituents X and Y, ring components U, V, W, T, and A<sup>1</sup> can be substituted in the pterioic acid reference structure while in most cases retaining folate receptor affinity.

15 An example of a preferred ligand according to the invention having a portion that is isosteric with pterioic acid is a ligand having formula I (including tautomers thereof) wherein

X and Y are each independently selected from the group consisting of hydrogen, halo, CH<sub>3</sub>, OH, SH and NH<sub>2</sub>, with X more preferably being OH;

20 U, V and W represent divalent moieties each independently selected from the group consisting of -CH= and -N= ;

A<sup>1</sup> is selected from the group consisting of -C(Z)-, -NH-, -N(CH<sub>3</sub>)-, -O-, -S-, -S(O)-, -S(O)<sub>2</sub>-, -CH<sub>2</sub>-, -CH(CH<sub>3</sub>)-, -C(CH<sub>3</sub>)<sub>2</sub>-, -N(CH<sub>2</sub>-C≡CH)- and -N(C≡CH)-; where Z is oxygen or sulfur;

25 R<sup>1</sup> is selected from the group consisting of hydrogen, halo and methyl;

R<sup>6</sup> and R<sup>7</sup> are each independently selected from the group consisting of hydrogen, halo, CH<sub>3</sub>, OH, SH and NH<sub>2</sub>; or, R<sup>6</sup> and R<sup>7</sup> are taken together to form O= ;

30 A<sup>2</sup> is selected from the group consisting of -C(Z)-, -C(Z)O-, -OC(Z)-, -N(R<sup>4</sup>)-, -C(Z)-N(R<sup>4</sup>)-, -N(R<sup>4</sup>)-C(Z)-, -O-C(Z)-N(R<sup>4</sup>)-, -N(R<sup>4</sup>)-C(Z)-O-,

$-N(R^{4'})-C(Z)-N(R^{5'})-$ ,  $-O-$ ,  $-S-$ ,  $-S(O)-$ ,  $-S(O)_2-$ ,  $-N(R^{4'})S(O)_2-$ ,  
 $-C(R^{6'}) (R^{7'})-$ ,  $C_1-C_6$  alkyl;  $C_1-C_6$  alkoxy; where Z is oxygen or sulfur;  
 p and r are each 1; and

T,  $R^{4''}$ ,  $R^{5''}$ ,  $R^{6''}$ ,  $R^{7''}$ , L, n, s and B are as defined above;

5 provided that the linker L does not include a naturally occurring amino acid covalently linked to  $A^2$  at its  $\alpha$ -amino group through an amide bond. More preferably, T is  $-C=C-$ .

A particularly preferred ligand of the invention is a derivative of pterioic acid and has formula I (including tautomers thereof) wherein X is OH; Y is  
 10  $NH_2$ ; U and W are each  $-N=$ ; V is  $-CH=$ ; T is  $-C=C-$ ;  $A^1$  is  $-NH-$ ;  $R^1$  is hydrogen;  $A^2$  is  $-C(O)-$ ,  $-C(O)O-$ , or  $-C(O)NH-$  and is *para* to  $A^1$ ;  $R^6$  and  $R^7$  are hydrogen; p, r and s are each 1; and L, n and B are as defined elsewhere herein; provided that the linker L does not include a naturally occurring amino acid covalently linked to  $A^2$  at its  $\alpha$ -amino group through an amide bond.

15 One embodiment of the invention is a ligand capable of binding to a folate recognition site, such as a folate binding protein, folate receptor, and the like. Such a non-peptide folate analog may also be described as a folate mimetic. Compounds illustrative of this embodiment are selected from the general formula I. Such analogs may operate as surrogates for folate in  
 20 methods utilizing folate, such as targeting molecules for cells or tissues expressing folate recognition sites.

In a particularly preferred embodiment, the compound of the invention has formula I and further exhibits binding affinity for a folate receptor. A relative binding affinity assay is described in detail in Example IV and in  
 25 Westerhoff et al. (Mol. Pharm., 1995, 48:459-471). Performing this assay is straightforward. A preferred compound exhibits a binding affinity for the folate receptor relative to folic acid of at least about 0.01, more preferably at least about 0.05, even more preferably at least about 0.10, even more preferably at least about 0.25, even more preferably at least about 0.50, and most preferably  
 30 at least about 0.75, wherein the binding affinity of folic acid for the folate receptor is defined as 1.0. It should be understood that the binding affinity of

the compound of the invention may exceed 1.0, in cases where the binding affinity of the compound for the folate receptor is greater than that of folic acid itself.

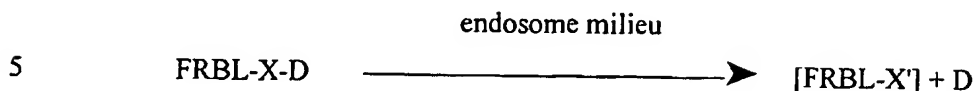
5 The compounds of formula I may optionally include a linker, spacer, or couple of variable length. The linker, spacer, or couple, hereinafter collectively referred to as a "linker," is adapted for connecting the folate analog to another molecule in other embodiments of the invention. A divalent linker L is present in the folate analog of formula I when the integer n is equal to 1. Such linkers are known in the art and are often used to "associate" one chemical entity to  
10 another. As used herein, the term "association" refers to any manner of coexistence of two or more molecules, such as complexation, chelation, ion-pairing, covalent bonding, and the like, such that for a time sufficient to administer the associated molecules, the associated molecules may be interpreted as a single entity.

15 The linker may create either a permanent or a semipermanent (i.e., labile) linkage. The inclusion of a semipermanent linkage is especially advantageous for applications in which cellular uptake of the drug is desired. The ability to form a bioactive conjugate utilizing a linkage other than a peptide linkage (e.g., the glutamyl linkage of typical folate conjugates) provides an  
20 important degree of chemical flexibility for the linkage of the pteric acid moiety to the drug payload. The capacity of a target cell for uptake of a folate-drug conjugate is expected to be dramatically increased when a linkage is selected that promotes drug release from the conjugate by exploiting known endosomal hydrolytic or reductive mechanisms (i.e., molecular separation  
25 between the drug payload and the ligand).

A preferred embodiment of the ligand-agent conjugate of the invention therefore includes a linker whereby a cell targeting ligand (i.e., the non-peptide folic acid analog) is chemically coupled to a drug molecule via a linker that is designed to be metabolized within the endosomal milieu. Following  
30 extracellular receptor binding and endocytic entry of the drug conjugate, endosome factors are expected to hydrolytically or reductively cleave the linker

moiety of the conjugate, thereby facilitating release of the drug from the ligand.

This process is depicted below:



wherein the abbreviations are as follows: FRBL, folate receptor binding ligand; X, endosome-cleavable linker; D, drug moiety; X', linker fragment.

10                      A preferred semi-permanent linker thus includes a functionality, such as a disulfide, ester, other hydrolyzable group, and the like, that allows separation of the ligand and the agent once the conjugate has reached the treatment site.

15                      Semi-permanent linkers preferably depend upon endogenous mechanisms of cleavage, and include metabolically labile linkers, such as a nucleotide, amide, ester, and the like subject to cleavage by peptidases, esterases, phosphodiesterases, reductases, and the like, which provides a stable ligand-agent conjugate prior to delivery but allows cleavage upon reaching the target or treatment site. Preferred linkers used to produce these drug conjugates are biologically labile (pH sensitive, redox sensitive, enzymatically sensitive) such that the ligand-receptor complex can be separated from the macromolecule

20                      "payload" in a predetermined manner (e.g., following endocytosis). The inclusion of a metabolically labile function is advantageously chosen in an end-use dependent manner such that following the binding of the conjugate, or additionally subsequent uptake of the conjugate as described below, the metabolically labile association may be cleaved thus releasing the agent from

25                      the ligand, either locally (extracellularly) in the case of binding of the conjugate to the cell surface, or intracellularly, as in the case of post-uptake by the cell.

30                      The divalent linker L comprises a linear or branched chain comprising a plurality of linking groups  $L_1, L_2, \dots, L_m$ , wherein "m" is an integer from 0 to about 50. Preferably, m is selected such the number of atoms in the linear backbone of linker L is at least about 1, more preferably at least about 3, most

preferably at least about 6; and at most about 100, more preferably at most about 50, and most preferably at most about 20.

Each linking group " $L_m$ " is also a divalent moiety composed of atoms selected from the group consisting of carbon, nitrogen, oxygen, and sulfur, providing that an oxygen atom is not adjacent to another oxygen or sulfur atom, except when the sulfur atom is oxidized, as in  $-S(O)_2-$ . Each individual linking unit " $L_m$ " can be the same or different and is thus independently selected from the group of divalent radicals. Illustrative divalent radicals are  $-CR^{6''}R^{7''}-$ ,  $-(R^{6''})C=C(R^{7''})-$ ,  $-CC-$ ,  $-C(O)-$ ,  $-O-$ ,  $-S-$ ,  $-SO_2-$ ,  $-N(R^{3''})-$ ,  $-(R^{6''})C=N-$ ,  $-C(S)-$ ,  $-P(O)(OR^{3''})-$ ,  $-P(O)(OR^{3''})O-$ , and the like.

$R^{3''}$  is a group suitable for nitrogen or oxygen attachment, such as hydrogen,  $C_1$ - $C_4$  alkyl,  $C_2$ - $C_4$  alkenyl,  $C_3$ - $C_8$  cycloalkyl, aryl,  $C_1$ - $C_4$  alkanoyl, aryloyl, and the like.  $R^{3''}$  attached to nitrogen may also be hydroxy,  $C_1$ - $C_4$  alkoxy, amino, monoalkylamino, or dialkylamino. It is appreciated that  $R^{3''}$  may be selected independently for each linking group  $L_m$ .

$R^{6''}$  and  $R^{7''}$  are each independently selected from groups suitable for carbon attachment such as hydrogen,  $C_1$ - $C_4$  alkyl,  $C_2$ - $C_4$  alkenyl, hydroxy, halo,  $C_1$ - $C_4$  alkoxy,  $C_3$ - $C_8$  cycloalkyl, aryl,  $C_1$ - $C_4$  alkanoyl, aryloyl, and the like. In addition,  $R^{6''}$  and  $R^{7''}$  are selected independently for each linking group  $L_m$ .

The linker L may also possess one or more cyclic regions, wherein a subset of the linking groups " $L_m$ " form one or more rings, including, but not limited to divalent cycloalkyl, such as cyclopent-1,3-diyl, cyclohex-1,1-diyl, and the like; divalent heterocyclyl, such as pyrrolidin-1,3-diyl, piperidin-2,2-diyl; and divalent aromatic groups, such as 1,3-phenylene, pyrrol-1,2-diyl, and the like.

Illustrative linkers L are polyalkylenes, polyalkylene glycols such as polyethylene glycol (PEG), N-(2-hydroxypropyl)methacrylamide (HPMA), and the like. Other examples of such linkers may be found in U.S. Patent Nos. 6,207,157, 6,184,042, 6,177,404, 6,171,859, and 6,171,614, the disclosures of which are incorporated herein by reference. The invention is not intended to be limited by the nature or length of the linker L.

The term "alkenyl" as used herein refers to a linear or branched chain of carbon atoms, such as ethenyl, propenyl, 2-methylethenyl, and the like.

The term "cycloalkyl" as used herein refers to a cyclic chain of carbon atoms, such as cyclopropyl, cyclopentyl, cyclohexyl, and the like.

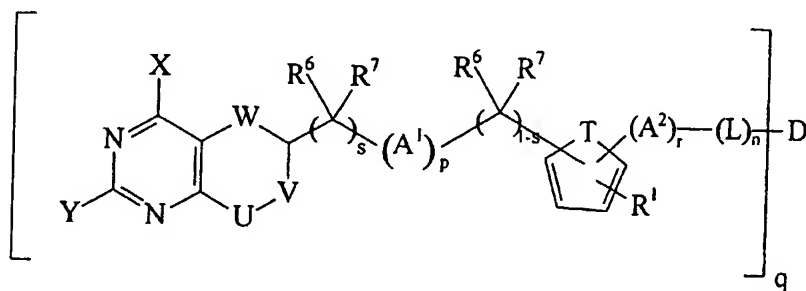
5 The term "aryl" as used herein refers to an aromatic moiety, such as phenyl, pyridinyl, pyrimidinyl, and the like. The aryl group is optionally substituted with from 1 to 3 substituents, such as with halo, alkyl, alkoxy, as defined above, and the like.

10 The term "aryloyl" as used herein refers to aryl, as defined above, substituted with a carbonyl group, such as benzoyl, picolinyl, and the like.

The term "polyalkylene" as used herein refers to polymers of alkenes, such as polyethylene, polypropylene, and the like.

15 Synthesis of non-peptide folic acid analogs may be accomplished by methods known to the skilled artisan. In addition, the optional incorporation of a linker may also be accomplished by methods known to the skilled artisan.

The present invention also provides a ligand-agent conjugate capable of binding to a folate recognition site, comprising a diagnostic or therapeutic agent in association with a non-peptide folic acid analog of general formula II:



20

## II

where X, Y, U, V, W, T, A<sup>1</sup>, A<sup>2</sup>, R<sup>1</sup>, R<sup>6</sup>, R<sup>7</sup>, L, n, p, r and s are as defined above;  
q is an integer  $\geq 1$ ; and,

D is a diagnostic agent or a therapeutic agent.



One embodiment of the invention is a ligand-agent conjugate capable of binding to a folate recognition site, such as a folate binding protein, folate receptor, and the like. Compounds illustrative of this embodiment are selected from the general formula II, where the integer q is equal to 1. Such analogs  
5 may operate as a means for targeting of and delivery to cells or tissues expressing folate recognition sites. The compounds of formula II may optionally include a linker L, where L is as defined above and where the integer n is equal to 1.

Another embodiment of the present invention is a ligand-agent  
10 conjugate capable of binding to a folate recognition site with high affinity, comprising a diagnostic or therapeutic agent in association with a plurality of non-peptide folic acid analogs of general formula II, where the integer q is 2 or greater. Similarly, such ligand-agent conjugates may optionally comprise a plurality of ligands each possessing a linker L, where L is as defined above and  
15 where the integer n is equal to 1. Such conjugates possessing a plurality of folate analogs in association with the diagnostic or therapeutic agent may advantageously enhance recognition of the conjugate by the recognition site.

The diagnostic or therapeutic agent D can be linked to the ligand at (L)<sub>n</sub> by any type of molecular interaction including a covalent bond, and ionic bond  
20 or association, hydrogen bonding or other type of complexation to form the ligand-agent conjugate.

Synthesis of ligand-agent conjugates may be accomplished by methods known to the skilled artisan depending upon the nature of the association of the ligand and the agent.

25 Virtually any type of molecule (small molecular weight chemotherapeutic, peptide, protein, oligosaccharide, antisense oligonucleotide, plasmid, ribozyme, artificial chromosome, micelle, liposome, etc.) can be more efficiently delivered into cells using this technology.

Diagnostic agents useful in the present invention include compounds  
30 capable of labeling a cell or tissue with a contrast agent for the generation or modulation of signal intensity in biomedical imaging. Such contrast agents

may be used for imaging such cells and tissues using techniques such as Magnetic Resonance Imaging (MRI), radio-imaging, radio-diagnosis, and the like. Such labeling of the cell or tissue is illustratively accomplished by incorporation of superparamagnetic, paramagnetic, ferrimagnetic, or ferromagnetic metals, radioactive gamma-emitting, positron-emitting or photon-emitting metals, radionuclides, other radioactive elements such as certain halogen isotopes (radiohalogens), and the like, in the agent. The diagnostic agent may be a chelating agent capable of binding such metals described above, or a radio-pharmaceutical possessing an organic fragment, such as an aromatic ring, possessing a radiohalogen. Such chelating agents are known to the skilled artisan.

Metals useful in the invention employing such chelating agents for MRI include certain ions of chromium, manganese, iron, cobalt, nickel, copper, praseodymium, neodymium, samarium, gadolinium, terbium, dysprosium, holmium, erbium, ytterbium, and the like, such as Cr(III), Mn(II), Fe(II), Fe(III), and Ni(II). Metals useful in the invention employing such chelating agents for radio-imaging include certain isotopes of gallium, indium, copper, technetium, rhenium, and the like, such as  $^{99m}\text{Tc}$ ,  $^{51}\text{Cr}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{103}\text{Ru}$ ,  $^{211}\text{Bi}$ ,  $^{64}\text{Cu}$  and  $^{111}\text{In}$ . Radiohalogens useful in the invention employing radio-pharmaceuticals include certain isotopes of fluorine, iodine, astatine, and the like, such as  $^{18}\text{F}$ ,  $^{123}\text{I}$ , and  $^{131}\text{I}$ .

Visualization techniques suitable for radioimaging are known in the art, such as positron emission tomography (PET), planar or SPECT imaging, gamma cameras, scintillation, and the like.

Therapeutic agents useful in the present invention include compounds capable of modifying, modulating, or otherwise altering certain cellular or tissue functions. Therapies include elimination of certain pathogenic cell populations or pathogenic tissues, enhancing beneficial functions in host cells or host tissues, protecting host cells or host tissues from non-selective treatment, and the like.

One embodiment of the invention is a ligand-therapeutic agent conjugate wherein the therapeutic agent targets pathogenic cells or tissues, such as tumors, bacteria, and the like. Such therapeutic agents include chemotherapeutic agents, antimicrobial agents, or other cytotoxic agents associated with the targeting ligand. Such cytotoxic agents, may lead to the destruction of the pathogenic cell or tissue. The therapeutic agent may be a radiotherapeutic agent. These agents, like the related diagnostic agents above, may possess a chelating functionality capable of sequestering a radionuclide, such as a radioactive metal or a radioactive alpha or beta-emitting metal suitable for nuclear medicine, or alternatively a suitable functionality bearing a radiohalogen, such as an aryl group. In this context however, the metal or halogen is used for radiotherapy rather than for radiodiagnosis. Metals appropriate for such radiotherapeutic agents are known in the art, including certain isotopes of gadolinium, technetium, chromium, gallium, indium, ytterbium, lanthanum, yttrium, samarium, holmium, dysprosium, copper, ruthenium, rhenium, lead, bismuth, and the like, such as  $^{157}\text{Gd}$ ,  $^{64}\text{Cu}$ ,  $^{67}\text{Cu}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{90}\text{Y}$ ,  $^{111}\text{In}$ , and  $^{177}\text{Lu}$ . Radiohalogens are also useful in the invention for radiotherapeutic agents, including certain isotopes of iodine, astatine, and the like, such as  $^{125}\text{I}$ ,  $^{131}\text{I}$ , and  $^{211}\text{At}$ . In another embodiment, the therapeutic may be a species suitable for neutron capture therapy, such as an organoborane moiety, comprising  $^{10}\text{B}$ .

Chemotherapeutic agents useful in the present invention include certain alkylating agents, such as busulfan, carboquone, chlomaphazine, lomustine, tubercidin, and the like, certain antimetabolites, such as fludarabine, doxifluridine, and the like, certain steroids and steroid analogs, such as calusterone, testolactone, flutamide, tamoxifen, hexestrol, melengestrol, and the like, certain antiadrenals, such as mitolane, and the like, certain LH-RH analogs, such as buserelin, leuprolide, and the like, and certain anti-angiogenic agents.

Another embodiment of the invention is therapeutic ligand-agent conjugate that targets cells or tissue, such that a beneficial function of the

targeted cell or tissue is enhanced by the therapeutic agent, such as an inflammatory, pro-inflammatory, or anti-inflammatory agent, antibiotic, analgesic, antiviral agent, and the like. Still other therapeutic agents useful in the present invention may protect a targeted cell or tissue from a subsequent  
5 non-selective treatment targeted to a different pathogenic cell or tissue, such as an immunosuppressant.

The present invention also provides a method for delivering a diagnostic or therapeutic agent to a targeted cell population. An effective amount of a ligand-agent conjugate comprising a diagnostic or therapeutic agent in  
10 association with a non-peptide folic acid analog of general formula II, where the integer q is 1 or greater, is delivered to the targeted cell population. The targeted cells possess a folate receptor to which the ligand-agent conjugate binds. The ligand thus selectively targets a certain cell or tissue, by binding to the receptors or proteins present in such cells or tissues that recognize the folic  
15 acid and folic acid analogs. If desired, a plurality of non-peptide folate analog conjugates can be administered.

In one embodiment of the method of the invention, the diagnostic or therapeutic effect is achieved as a direct or indirect result of binding of the ligand-agent conjugate to the folate receptor on the cell surface (i.e.,  
20 "docking"). For example, in vivo biomedical imaging can be facilitated whether or not the diagnostic agent is internalized, and in some instances, for example in the case of a cytotoxic diagnostic agent, it is preferable that the diagnostic agent remain outside the cell. As another example, the therapeutic agent can include an immune stimulating factor such as an antigen, which is  
25 preferably retained on the extracellular surface on the cell.

In another embodiment of the method of the invention, the diagnostic or therapeutic effect is achieved as a result of uptake or internalization of the therapeutic or diagnostic agent via binding to the folate receptor followed by internalization of the receptor-ligand complex. It is appreciated that the method  
30 of the invention is suitable for effecting uptake by cells or tissue of ligand-agent conjugates, where the agent is a molecule or compound that would otherwise

exhibit poor uptake by the cell or tissue by active transport, diffusion, or other passive transport.

The targeted cell population can be endogenous to exogenous to the patient. For example, it can be an endogenous population comprising a somatic  
5 or tumor cell population in a patient, a cancerous cell population, an organ, tissue or bodily fluid, or a virus-infected cell population. The ligand-agent conjugate can be delivered to a patient locally or systemically. For example, the conjugate can be delivered parenterally by intramuscular, intravenous or subcutaneous injection; likewise it can be formulated for oral administration.

10 An exogenous population of cells can include an ex vivo or in vitro population of cells. For example, the target cell population can be an ex vivo population of cells such as bone marrow cells, stem cells, or cells of an organ or tissue that have been removed from the patient's body. The ex vivo cells are contacted with the ligand-agent conjugate of the invention and subsequently  
15 returned to the body of the patient. Gene therapy, for example, can be accomplished using a ligand-agent conjugate of the invention wherein the therapeutic agent is a nucleic acid.

Likewise the target population can be an in vitro population of cells such as a tissue or fluid sample or biopsy that has been removed from a patient  
20 for diagnostic purposes. The biological sample can be contacted with the ligand-agent conjugate of the invention comprising a diagnostic agent for detection or characterization of the disease state of the patient.

An exogenous population of cells can also include a population of exogenous organisms such as bacteria, mycoplasma, yeast, or fungi, provided  
25 the organisms possess a receptor molecule that binds the ligand-agent conjugate. See, e.g., Kumar et al., 1987, *J. Biol. Chem.* 262(15):7171-9. The ligand-agent conjugate binds to the surface of the tumor cells or pathogenic organisms and "labels" or otherwise alters or modifies cell or tissue function; it may or may not be internalized, depending on the intended application.

30

## EXAMPLES

The following examples are illustrative of certain embodiments of the invention. The examples, methods, and conditions presented therein are not to be construed as limiting the scope nor the spirit of the invention.

5

## Example I.

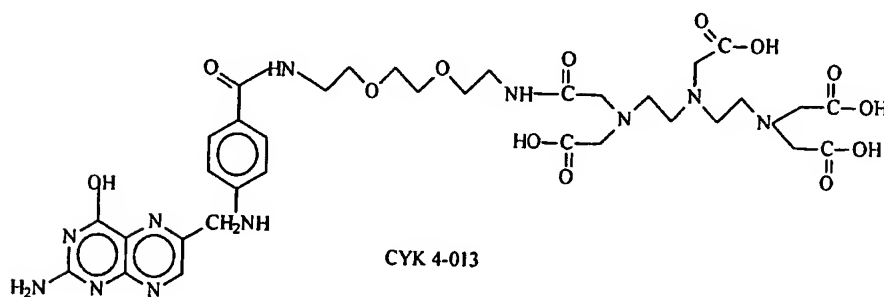
Targeting the Tumor-associated Folate Receptor with a  $^{111}\text{In}$ -DTPA Conjugate of Pterioic Acid

*Objective.* The present study was undertaken to evaluate the structural requirements for folate-receptor-targeting with low-molecular-weight radiometal chelates, specifically examining the role of the amino acid fragment of folic acid (pteroyl-glutamic acid) in mediating folate-receptor affinity.

*Methods.* The amide-linked conjugate pteroyl-NHCH<sub>2</sub>CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>NH-DTPA (CYK4-013), which lacks an amino acid in the linker region, was prepared by a three-step procedure from pterioic acid, 2,2'-(ethylenedioxy)-bis(ethylamine), and t-Bu-protected DTPA.

20

25



This conjugate (CYK4-013) was prepared as outlined in Figure 2. CY3-064 was obtained from pterioic acid (0.025 g; 0.080 mmol) and a large excess of 2,2'-(ethylenedioxy)-bis(ethylamine) (0.237 g; 1.6 mmol) using the coupling reagents. Benzotriazole-1-yl-oxy-tris(pyrrolidino)phosphonium

30

hexafluorophosphate (PyBOP) (0.125 g; 0.24 mmol), N-hydroxybenzotriazole (HOBt) (0.037 g; 0.24 mmol), and N-methylmorpholine (Nmm)(0.049 g; 0.48 mmol) in dry dimethylsulfoxide (DMSO) (0.8 mL) at room temperature for 22 hours under nitrogen. The excess reagent and solvent DMSO were removed  
5 under vacuum and the resulting brown residue triturated with diethylether, methanol, and water to produce 20 mg of CY3-064 as a yellow solid (57% estimated yield).

This yellow solid was then coupled with t-butyl-protected DTPA (synthesized as described by S.A. Chilefu, *et al.*, *J. Org. Chem.*, 2000, 65:1562-  
10 1565) using the same coupling reagents (0.012 g CY3-064; 0.071 g t-Bu-DTPA, 0.127 mmol; 0.0848 g PyBOP, 0.16 mmol; 0.0249 g HOBt, 0.16 mmol; 0.0247 g Nmm, 0.24 mmol; 0.6 mL dry DMSO). During coupling, the solubility of CY3-064 was very poor in the DMSO solvent, and not improved by addition of more Nmm. 1, 3, 4, 6, 7, 8-hexahydro-1-methyl-2H-pyrimido[1,2-a]-  
15 pyrimidine (MTBD) (0.0083 g; 0.0542 mmol) was added after stirring overnight at room temperature, but the solubility remained poor. After again stirring overnight, the DMSO and excess reagents were removed under high vacuum overnight.

The resulting brown residue was triturated first with diethylether and  
20 then with methanol. The methanol suspension was centrifuged to produce crude CY3-078 as a yellow solid. This yellow solid was purified using semi-preparative HPLC (2x) on a C18 column (10 x 250 mm) to produce pure CY3-078. (HPLC solvent A = 5% CH<sub>3</sub>CN in 0.1% aqueous TFA; solvent B = 10% water in 0.1% TFA in CH<sub>3</sub>CN. Linear gradients established as: 5% B at time =  
25 zero ramping to 70% B at 30 minutes, then ramping to 100% B at 32 minutes, and remaining 100% B to 40 minutes. Flow rate = 2.35 mL/min). The peak with retention time of 30.5 minutes was collected. The purified CY3-078 was treated with 70% TFA/CH<sub>2</sub>Cl<sub>2</sub> at 0°C for 30 minutes, and stirred at room  
30 temperature for 5 hours, to remove the three t-Bu protecting groups. The resulting CYK4-013 product (6 mg) was isolated by trituration with diethylether. Both CY3-078 and CYK4-013 exhibit the expected parent ion

peaks in their positive and negative ion electrospray mass spectra. In particular, purified CYK4-013 exhibited the expected parent ion peaks in its positive and negative ion electrospray mass spectra ( $m/e = 818$  and  $816$ , respectively).

5 The  $^{111}\text{In}$  complex of CYK4-013 was prepared from  $^{111}\text{In}$ -chloride (1.2 mCi; 44 MBq) and purified by reversed-phase HPLC. Specifically, the  $^{111}\text{In}$  complex of CYK4-013 was prepared from  $^{111}\text{In}$ -chloride via ligand exchange in acetate buffer. Briefly, 1.2 mCi no-carrier-added  $^{111}\text{In}$ -chloride (Mallinckrodt, Inc., St. Louis) in 0.05 mL 0.05 N HCl was transferred to a small tube and 0.05 mL 0.1N ammonium acetate (pH 5.5), followed by 0.02 mL 0.5N  
10 ammonium acetate (pH 7.4) was added, producing a solution with pH 7. The CYK4-013 ligand was weighed out and diluted in water, pH adjusted with 1N NaOH to pH 9-10. 10  $\mu\text{L}$  of this ligand solution containing 95  $\mu\text{g}$  CYK4-013 was added to the  $^{111}\text{In}$ -acetate solution (120  $\mu\text{L}$ ) and mixed. The solution was protected from light and kept at room temperature.

15 The radiochemical purity of the resulting crude  $^{111}\text{In}$ -CYK4-013 was evaluated by radio-HPLC using a 4.6 x 250 mm Dynamax C18 column (Varian/Rainin) eluted with an aqueous  $\text{NH}_4\text{OAc}$ :acetonitrile gradient. HPLC conditions: Solvent A = 56 mM  $\text{NH}_4\text{OAc}$  in water; Solvent B =  $\text{CH}_3\text{CN}$ . Flow rate = 1 mL/min on 4.6 x 250 mm C18 reverse-phase column. Linear gradient  
20 conditions: 5% B at zero minutes, ramping to 25% B at 25 minutes, then ramping to 60% B at 27 minutes and 100% B at 30 minutes).

The major radioactive HPLC peak, eluting with a retention time of 14.1 minutes, was collected. HPLC analysis of this isolated peak showed it to remain stable for at least 7 days at room temperature. HPLC conditions:  
25 Solvent A = 56 mM  $\text{NH}_4\text{OAc}$  in water; Solvent B =  $\text{CH}_3\text{CN}$ . Flow rate = 1 mL/min on 4.6 x 250 mm C18 reverse-phase column. Linear gradient conditions: 5% B at zero minutes, ramping to 25% B at 25 minutes, then ramping to 60% B at 27 minutes and 100% B at 30 minutes.

30 Radio-TLC of the HPLC-purified  $^{111}\text{In}$ -CYK4-013 was performed using a C18 plate eluted with 25%  $\text{NH}_4\text{OAc}$ ; 75% acetonitrile. The results confirm



the absence of  $^{111}\text{In}$ -species that irreversibly adsorb to C18 (i.e., there is no  $^{111}\text{In}$  remaining at origin)

The HPLC-purified  $^{111}\text{In}$ -CYK4-013 was removed from the HPLC solvents by solid-phase extraction. A C18 Sep-Pak Light solid phase extraction cartridge (Millipore, Inc.) was conditioned by washing with ethanol followed  
5 by water. The  $^{111}\text{In}$ -CYK4-013 was loaded onto the C18 Sep-Pak after dilution with water to 5% acetonitrile, the Sep-Pak was washed with 3 mL water, and the  $^{111}\text{In}$ -CYK4-013 product was recovered by fractional elution with ethanol. The resulting ethanol solution of  $^{111}\text{In}$ -CYK4-013 was evaporated to dryness  
10 under a stream of  $\text{N}_2$  at room temperature, and the  $^{111}\text{In}$ -CYK4-013 was reconstituted in saline for use in a biodistribution study in mice.

Our primary animal model for evaluation of the biodistribution and pharmacokinetics of folate-receptor-targeted radiopharmaceuticals has been athymic mice bearing subcutaneously implanted folate-receptor-positive human  
15 KB cell tumors. Because normal rodent chow contains a high concentration of folic acid (6 mg/kg chow), the mice used in these receptor targeting studies were maintained on folate-free diet for 3 weeks to achieve serum folate concentrations close to the 4-6  $\mu\text{g/L}$  (9-14 nM) range of normal human serum. After 3 weeks on folate-free diet, mouse serum folate levels drop to  $25 \pm 7$  nM  
20 from the initial  $720 \pm 260$  nM serum folate level when the animals are fed normal rodent chow. This dietary intervention is believed to be a reasonable manipulation of the animal model, since the mice would have serum folate levels only slightly higher than the folate concentration of normal human serum. Thus, in these mouse biodistribution studies the radiotracer is  
25 competing for tumor folate receptors with physiologically relevant concentrations of endogenous unlabeled serum folate.

Thus, to demonstrate the ability of such conjugates to selectively localize in folate-receptor-positive tissues, the biodistribution of  $^{111}\text{In}$ -CYK4-013 was determined following intravenous administration to athymic mice with  
30 subcutaneous folate-receptor-positive human KB cell tumor xenografts. The resulting data are presented in Tables 1 and 2. The  $^{111}\text{In}$ -CYK4-013 agent is

found to selectively localize in the folate-receptor-positive tumors ( $5.4 \pm 0.8$  and  $5.5 \pm 1.1$  percent of the injected  $^{111}\text{In}$  dose per gram of tumor at 1 hour and 4 hours post-injection, respectively) and to exhibit prolonged tumor retention of the radiolabel ( $3.6 \pm 0.6$  percent of the injected  $^{111}\text{In}$  dose per gram of tumor still remaining at 24 hours post-injection). The tumor localization of the  $^{111}\text{In}$ -radiolabel clearly appears to be mediated by the cellular folate receptor, since the tumor uptake of radiotracer drops precipitously ( $0.12 \pm 0.07$  percent of the injected  $^{111}\text{In}$  dose per gram at 4 hours) when  $^{111}\text{In}$ -CYK4-013 is co-injected with an excess of folic acid, which will compete for folate receptor sites.

Urinary excretion appears to be the primary whole-body clearance pathway for the  $^{111}\text{In}$ -CYK4-013. The substantial retention of  $^{111}\text{In}$  in the kidneys is fully consistent with the binding of  $^{111}\text{In}$ -CYK4-013 to tissue folate receptors, since the renal proximal tubule is a known normal tissue site of folate receptor expression. This interpretation is supported by the expected and observed marked reduction in renal  $^{111}\text{In}$  when  $^{111}\text{In}$ -CYK4-013 is co-administered with excess folic acid. The behavior of the  $^{111}\text{In}$ -CYK4-013 radiopharmaceutical in this animal model (Table 2) is very similar to that observed for  $^{111}\text{In}$ -DTPA-Folate (Table 3).

*Results.* Biodistribution of  $^{111}\text{In}$ -CYK4-013 is shown in Tables 1-3.

Similar to  $^{111}\text{In}$ -DTPA-Folate,  $^{111}\text{In}$ -CYK4-013 selectively localized in the folate-receptor-positive tumor xenografts, and afforded prolonged tumor retention of  $^{111}\text{In}$  ( $5.4 \pm 0.8$ ;  $5.5 \pm 1.1$ ; and  $3.6 \pm 0.6$  %ID/g at 1 hour, 4 hours, and 24 hours, respectively) (Table 2). The tumor localization of the  $^{111}\text{In}$ -radiolabel appears to be mediated by the cellular folate receptor, since the tumor uptake dropped precipitously ( $0.12 \pm 0.07$  %ID/g at 4 hours) when  $^{111}\text{In}$ -CYK4-013 was co-injected with an excess of folic acid (Table 2). Blockable binding was also observed in the kidneys, where the folate receptor occurs in the proximal tubules.

Table 1  
Biodistribution of  $^{111}\text{In}$ -CYK4-013 in KB Tumor-Bearing Athymic Mice  
at Various Times Following Intravenous Administration

	Percentage of Injected $^{111}\text{In}$ Dose Per Organ (Tissue)			
	1 Hour	4 Hours	4 Hours — Blocked**	24 Hours
<i>Tumormass (g):</i>	$0.15 \pm 0.10$	$0.080 \pm 0.031$	$0.077 \pm 0.010$	$0.104 \pm 0.097$
<i>Animalmass (g):</i>	$29.5 \pm 1.2$	$28.6 \pm 1.6$	$28.2 \pm 1.1$	$28.6 \pm 0.8$
<i>Animal Quantity &amp; Gender:</i>	3M	4M	4M	4M
<b>Blood:</b>	$0.29 \pm 0.003$	$0.078 \pm 0.010$	$0.025 \pm 0.015$	$0.055 \pm 0.002$
<b>Heart:</b>	$0.51 \pm 0.09$	$0.41 \pm 0.08$	$0.0023 \pm 0.0016$	$0.18 \pm 0.06$
<b>Lungs:</b>	$0.69 \pm 0.06$	$0.60 \pm 0.01$	$0.0082 \pm 0.0051$	$0.34 \pm 0.03$
<b>Liver &amp; Gall Bladder:</b>	$6.8 \pm 1.3$	$3.0 \pm 1.0$	$0.074 \pm 0.044$	$1.7 \pm 0.9$
<b>Spleen:</b>	$0.063 \pm 0.016$	$0.049 \pm 0.012$	$0.0049 \pm 0.0029$	$0.055 \pm 0.017$
<b>Kidney (one):</b>	$15.3 \pm 1.2$	$20.2 \pm 1.6$	$0.23 \pm 0.15$	$26.8 \pm 2.7$
<b>Stomach, Intestines &amp; Contents:</b>	$5.8 \pm 0.5$	$5.4 \pm 0.8$	$4.6 \pm 2.8$	$3.3 \pm 0.6$
<b>Muscle:</b>	$43.6 \pm 4.1$	$33 \pm 9$	$1.0 \pm 0.7$	$23.1 \pm 7.0$
<b>Tumor:</b>	$0.78 \pm 0.47$	$0.46 \pm 0.26$	$0.0094 \pm 0.0061$	$0.42 \pm 0.4$

\*Athymic mice (NuNu strain) with subcutaneous tumors. Born 9/11/00. Arrived 10/10/00. Initiated folate-free diet 10/10/00. Implanted on 10/20/00;  $0.25 \times 10^6$  KB cells (passage 9) per animal subcutaneous in intrascapular region. Study date: November 2, 2000. Values shown represent the mean  $\pm$  standard deviation. Blood was assumed to account for 5.5% of total body mass. Muscle was assumed to account for 42% of the total body mass.

\*\*Folate receptors blocked by co-injection of folic acid dihydrate at a dose of  $4.1 \pm 0.4$  mg/kg.

Table 2  
Biodistribution of  $^{111}\text{In}$ -CYK4-013 in KB Tumor-Bearing Athymic Mice  
at Various Times Following Intravenous Administration

5		Percentage of Injected $^{111}\text{In}$ Dose Per Gram (Tissue Wet Mass)			
		1 Hour	4 Hours	4 Hours — Blocked**	24 Hours
	<i>Tumormass (g):</i>	$0.15 \pm 0.10$	$0.080 \pm 0.031$	$0.077 \pm 0.010$	$0.104 \pm 0.097$
	<i>Animalmass (g):</i>	$29.5 \pm 1.2$	$28.6 \pm 1.6$	$28.2 \pm 1.1$	$28.6 \pm 0.8$
10	<i>Animal Quantity &amp; Gender:</i>	<i>3M</i>	<i>4M</i>	<i>4M</i>	<i>4M</i>
	Blood:	$0.18 \pm 0.01$	$0.050 \pm 0.009$	$0.016 \pm 0.010$	$0.035 \pm 0.002$
	Heart:	$3.5 \pm 0.4$	$2.9 \pm 0.9$	$0.015 \pm 0.010$	$1.2 \pm 0.5$
15	Lungs:	$1.5 \pm 0.2$	$1.4 \pm 0.3$	$0.041 \pm 0.027$	$0.72 \pm 0.21$
	Liver & Gall Bladder:	$4.3 \pm 0.7$	$1.9 \pm 0.6$	$0.051 \pm 0.029$	$1.2 \pm 0.6$
	Spleen:	$0.31 \pm 0.09$	$0.29 \pm 0.07$	$0.028 \pm 0.017$	$0.27 \pm 0.08$
	Kidney (one):	$61 \pm 5$	$81 \pm 7$	$0.90 \pm 0.59$	$105 \pm 7$
20	Stomach, Intestines & Contents:	$1.7 \pm 0.2$	$1.5 \pm 0.9$	$1.7 \pm 1.1$	$1.2 \pm 0.2$
	Muscle:	$3.5 \pm 0.5$	$2.8 \pm 0.8$	$0.080 \pm 0.057$	$1.9 \pm 0.6$
	Tumor:	$5.4 \pm 0.8$	$5.6 \pm 1.1$	$0.12 \pm 0.07$	$3.6 \pm 0.6$
25	<i>Tumor/blood</i>	$30 \pm 5$	$111 \pm 11$	$7.5 \pm 2.4$	$105 \pm 20$
	<i>Tumor/kidney</i>	$0.088 \pm 0.013$	$0.069 \pm 0.013$	$0.12 \pm 0.03$	$0.035 \pm 0.008$
	<i>Tumor/liver</i>	$1.3 \pm 0.4$	$3.0 \pm 0.5$	$2.2 \pm 0.6$	$3.7 \pm 1.4$
	<i>Tumor/muscle</i>	$1.6 \pm 0.4$	$2.1 \pm 0.2$	$1.5 \pm 0.7$	$2.0 \pm 0.6$

\*Athymic mice (NuNu strain) with subcutaneous tumors. Values shown represent the mean  $\pm$  standard deviation.

\*\*Folate receptors blocked by co-injection of folic acid dihydrate at a dose of  $4.1 \pm 0.4$  mg/kg.

Table 3  
Biodistribution of  $^{111}\text{In}$ -DTPA-Folate in Athymic Mice  
with Subcutaneous KB Cell Tumor Xenografts

5		Percentage of Injected $^{111}\text{In}$ Dose Per Gram (mean $\pm$ s.d.; n=4)		
		1 Hour Post Injection	4 Hours Post Injection	4 Hours Post Injection BLOCKED
	<i>Tumormass (g):</i>	<i>0.138<math>\pm</math>0.051</i>	<i>0.202<math>\pm</math>0.083</i>	<i>0.193<math>\pm</math>0.078</i>
	<i>Animalmass (g):</i>	<i>24<math>\pm</math>2</i>	<i>25<math>\pm</math>1</i>	<i>24<math>\pm</math>1</i>
	<i>Folic Acid Dose (<math>\mu\text{g/kg}</math>):</i>	<i>0</i>	<i>0</i>	<i>495<math>\pm</math>79</i>
10	Blood:	0.14 $\pm$ 0.03	0.064 $\pm$ 0.007	0.029 $\pm$ 0.011
	Heart:	2.3 $\pm$ 0.4	2.0 $\pm$ 0.3	0.022 $\pm$ 0.010
	Lungs:	1.3 $\pm$ 0.1	1.1 $\pm$ 0.3	0.065 $\pm$ 0.021
15	Liver & Gall Bladder:	4.0 $\pm$ 1.5	2.2 $\pm$ 0.4	0.12 $\pm$ 0.03
	Spleen:	0.36 $\pm$ 0.03	0.35 $\pm$ 0.11	0.060 $\pm$ 0.021
20	Kidney:	90 $\pm$ 9	85 $\pm$ 12	2.3 $\pm$ 1.0*
	Stomach, Intestines & Contents:	1.0 $\pm$ 0.2	1.0 $\pm$ 0.2	0.49 $\pm$ 0.20
25	Muscle:	3.5 $\pm$ 0.8	2.9 $\pm$ 0.7	0.023 $\pm$ 0.013
	Tumor:	5.3 $\pm$ 0.4	6.8 $\pm$ 1.2	0.16 $\pm$ 0.07
30	<i>Tumor/blood</i>	<i>38<math>\pm</math>7</i>	<i>106<math>\pm</math>15</i>	<i>5.5<math>\pm</math>0.8</i>
	<i>Tumor/kidney</i>	<i>0.060<math>\pm</math>0.011</i>	<i>0.080<math>\pm</math>0.012</i>	<i>0.050<math>\pm</math>0.023</i>
	<i>Tumor/liver</i>	<i>1.5<math>\pm</math>0.5</i>	<i>3.3<math>\pm</math>1.1</i>	<i>1.4<math>\pm</math>0.4</i>
35	<i>Tumor/muscle</i>	<i>1.6<math>\pm</math>0.3</i>	<i>2.5<math>\pm</math>0.9</i>	<i>8.4<math>\pm</math>3.8</i>

\*n = 3 (While 4 animals were studied, one gave an unusually high value for the kidney uptake with no apparent underlying cause for the disparity with the other animals in this group. If that anomalous value is included, this result becomes 5.0  $\pm$  5.5 %ID/g, n = 4).

*Conclusion.* Tumor-selective drug targeting via the folate receptor remains feasible with pterioic acid conjugates lacking amino acid fragments, such as the glutamic acid moiety of folic acid.

5

### Example II.

#### Synthesis of a DOTA Conjugate of Pterioic Acid

A pterioic acid conjugate linked to the tetraazamacrocyclic DOTA  
10 chelating ligand was prepared for radiolabeling with radiometals such as  $^{64}\text{Cu}^{2+}$  and  $^{111}\text{In}^{3+}$ . This conjugate (CY4-036) was prepared as shown in Figure 3. The starting material for the synthesis was pterioic acid. Due to the poor solubility of pterioic acid in organic solvent, pterioic acid was protected with 2-(trimethylsilyl)ethanol to produce intermediate CY4-033 using literatural  
15 procedure (M. Nomura, et al., *J. Org. Chem.*, 2000, 65, 5016-5021) to increase its solubility in organic solvent before coupling to the DOTA derivative.

DOTA was coupled to 2,2'-(ethylenedioxy)bis(ethyleneamine) using PyBOP, HOBt, and NMM as coupling reagents in DMF to produce CY4-032. Protected form of pteroyl-linker-DOTA (CY4-034) was obtained through the  
20 coupling of CY4-032 and CY4-033 in DMSO using MTBD as a base, and then purified via flash chromatography eluted with gradient MeOH/ $\text{CHCl}_3$ .

All the protecting groups (three t-butyl groups on carboxylic acids and one 2-(trimethylsilyl)ethyloxycarbonyl on nitrogen) were removed by the treatment with 70% TFA/ $\text{CH}_2\text{Cl}_2$  to produce CY4-036.

25 *Synthesis of CY4-032.* To a solution of DOTA-tri-t-butyl ester (0.050 g, 0.087 mmol), PyBOP (0.136 g, 0.261 mmol), and HOBt (0.052 g, 0.339 mmol) in DMF (0.9 mL) was added NMM (0.035 g, 0.348 mmol) under  $\text{N}_2$ . The clear solution was stirred at room temperature (i.e., about 25°C) for 10 minutes followed by the addition of 2,2'-(ethylenedioxy)bis(ethyleneamine) (0.065 g, 0.436 mmol) under  $\text{N}_2$ . After stirring at room temperature for 17 h, the solution  
30 was concentrated under high vacuum to remove DMF and excess reagents. The

oily residue was triturated with Et<sub>2</sub>O (3 x 5 mL). After the removal of Et<sub>2</sub>O, EtOAc (3 mL) was added followed by 2 mL of water. After stirring for 2 minutes, the EtOAc was separated from aqueous layer and then more EtOAc (3 mL) was added for the extraction of product. The extraction was repeated one  
5 more time. All three EtOAc layers were combined, concentrated, and then dried under high vacuum to produce 0.153 g of oily crude product. C<sub>34</sub>H<sub>66</sub>N<sub>6</sub>O<sub>9</sub> = 702; Electrospray (+): M+H 703. This crude material was used for the next coupling step without further purification.

*Synthesis of CY4-033.* To a suspension of carbonyl diimidazole (CDI)  
10 (0.069 g, 0.425 mmol) and pterioic acid (0.028 g, 0.090 mmol) in DMSO (0.8 mL) was added triethylamine (0.032 g, 0.32 mmol) under N<sub>2</sub>. After stirring at room temperature for 3.5 hours, 2-(trimethylsilyl)ethanol (0.076 g, 0.64 mmol) was added and stirred at room temperature for 5.5 hours. The reaction mixture was concentrated under high vacuum overnight to remove DMSO and excess  
15 reagents. Yellow residue was produced and triturated with Et<sub>2</sub>O. A yellow solid (0.067 g) was produced as crude product. This crude material was used for next reaction without further purification.

*Synthesis of CY4-034.* To a solution of CY4-032 (0.153 g) and CY4-033 (0.067 g) in DMSO (0.8 mL) was added MTBD (0.041 g, 0.27 mmol) under N<sub>2</sub>.  
20 After stirring at room temperature for 21 hour, the reaction mixture was dried under high vacuum to remove DMSO and excess reagents to produce 0.157 g of yellow residue. This crude product was purified via flash chromatography eluted with gradient MeOH/CHCl<sub>3</sub> to produce 0.073 g of pure CY4-034.

*Synthesis of CY4-036.* 70%TFA/CH<sub>2</sub>Cl<sub>2</sub> (1 mL) was added to the purified  
25 CY4-034 (0.018 g) at room temperature. After stirring at room temperature for 4.5 hour, the reaction mixture was concentrated under reduced pressure and dried under high vacuum overnight to produce 19 mg of crude product. All three t-Butyl groups and the 2-(trimethylsilyl)ethyloxycarbonyl protecting group were removed at this step.

30 *<sup>64</sup>Cu-complex of CY4-036.* The <sup>64</sup>Cu complex of CY4-036 was prepared from <sup>64</sup>Cu-chloride via ligand exchange in acetate buffer. Briefly, 2.88 mCi of

no-carrier-added  $^{64}\text{Cu}$ -chloride (Washington University, St. Louis, MO) in 0.005 mL of 0.01 N HCl was transferred to a small test tube and mixed with 0.010 mL of 0.5 M ammonium acetate (pH 7.4). The CY4-036 ligand was weighed out and diluted in water and the pH adjusted with 1 N NaOH to pH 11-12. One  $\mu\text{L}$  of this ligand solution containing  $\sim 125 \mu\text{g}$  CY4-036 was added to the  $^{64}\text{Cu}$ -acetate solution and mixed. The pH of the solution was adjusted to pH 8-9 with the addition of 1  $\mu\text{L}$  of 1 N NaOH. The solution was protected from light and incubated at  $65^\circ\text{C}$  for 30 minutes.

The resulting crude  $^{64}\text{Cu}$ -CY4-036 was diluted with water and injected onto radio-HPLC using a 10 x 250 mm Dynamax C18 column (Varian/Rainin) eluted with an aqueous  $\text{NH}_4\text{OAc}$ :acetonitrile gradient. HPLC conditions: Solvent A = 56 mM  $\text{NH}_4\text{OAc}$  in water; Solvent B =  $\text{CH}_3\text{CN}$ . Flow rate = 2.35 mL/min on 10 x 250 mm C18 reverse-phase column. Linear gradient conditions: 5% B at zero minutes, ramping to 25% B at 25 minutes, then ramping to 60% B at 27 minutes and 100% B at 30 minutes. The major radioactive HPLC peak, eluting with a retention time of 20.9 minutes, was collected. Radio-TLC confirmed the absence of  $^{64}\text{Cu}(\text{II})$ -acetate, which was independently shown to remain at the origin.

HPLC-purified  $^{64}\text{Cu}$ -CY4-036 was removed from the HPLC solvents by solid-phase extraction. A C18 Sep-Pak Light solid phase extraction cartridge (Millipore, Inc.) was conditioned by washing with ethanol followed by water. The HPLC-purified  $^{64}\text{Cu}$ -CY4-036 was loaded onto the C18 Sep-Pak after dilution with water to 5% acetonitrile, the Sep-Pak was washed with 20 mL water, and the  $^{64}\text{Cu}$ -CY4-036 product recovered by fractional elution with ethanol. The resulting ethanol solution of  $^{64}\text{Cu}$ -CY4-036 was evaporated to dryness under a stream of  $\text{N}_2$  at room temperature, and the  $^{64}\text{Cu}$ -CY4-036 was reconstituted in water. Analytical HPLC confirmed the radiochemical purity, and stability, of the isolated  $^{64}\text{Cu}$ -CY4-036 product. HPLC conditions: Solvent A = 53 mM  $\text{NH}_4\text{OAc}$  in water; Solvent B =  $\text{CH}_3\text{CN}$ . Flow rate = 1 mL/min on 4.6 x 250 mm C18 reverse-phase analytical column. Linear gradient conditions: 5% B



at zero minutes, ramping to 25% B at 25 minutes, then ramping to 60% B at 27 minutes and 100% B at 30 minutes.

### Example III.

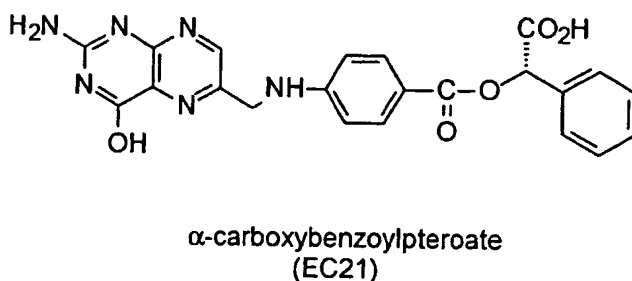
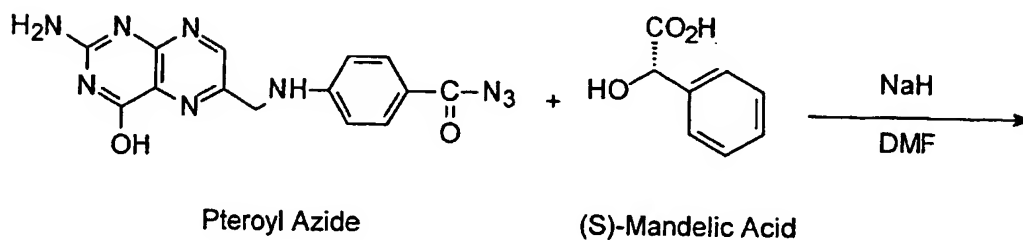
#### 5 Mandelic Acid Conjugate of Pterioic Acid

Biodegradation of an ester formed from pterioic acid and a substituted derivative of mandelic acid is shown in Figures 4 and 5. Figure 4 illustrates bioreduction of the conjugate to release the drug or drug-bearing moiety, while  
10 Figure 5 illustrates acid hydrolysis of the conjugate to release the drug or drug-bearing moiety. Evidence from a recent article describing structure-activity relationships of the folate receptor suggests that the proton on the nitrogen which forms part of the amide bond between pterioic acid and glutamic acid is not necessary for high-affinity binding (Westerhof et al., 1995, *Molecular*  
15 *Pharmacology* 48, 459-471). Accordingly, it is highly anticipated that the mandelate esters depicted in these schemes will bind with high affinity to the folate receptor. An example is shown in Example IV.

## Example IV.

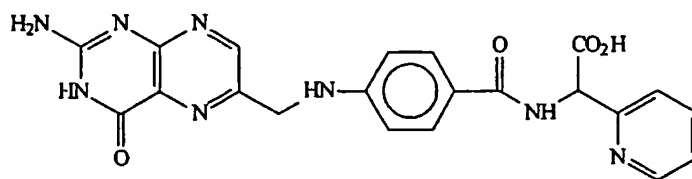
Synthesis and Activity of (S)- $\alpha$ -carboxybenzoyl pteroate (ACBP) and N-pteroyl-2-amino-2-carboxymethylpyridine (Pte-AP)

5



10      *Synthesis of ACBP.* (S)- $\alpha$ -carboxybenzoyl pteroate (ACBP) is a  
 mandelate ester (see Example III). A solution of (S)-mandelic acid (28 mg,  
 0.187 mmol) in 2 mL of anhydrous dimethylformamide was added via syringe to  
 30 mg of a 60% dispersion of NaH in mineral oil (under argon). After stirring for  
 15      10 minutes at room temperature, solid pteroyl azide (64 mg, 0.187 mmol) was  
 added and the reaction was stirred for an additional 2 hours. The reaction was  
 quenched with a solution of 50 mg  $\text{NH}_4\text{Cl}$  in 60 mL of deionized water. The  
 resulting solution was washed with hexanes (to remove the mineral oil) and then  
 diethylether. The aqueous solution was sparged with argon while the flask was  
 15      immersed in warm water to evaporate the residual diethylether. The solution was  
 brought to pH 2.2 by drop-wise addition of 1 N HCl, whereupon the product

precipitated as a yellow-orange finely-divided solid. The solid was isolated by centrifugation and washed twice with deionized water. The material was dissolved in 6 mL of deionized water containing  $\text{NH}_4\text{HCO}_3$  (16 mg, 0.2 mmol). The resulting solution was filtered and then purified by HPLC: Novapak 19 x 300 mm prep column, gradient 0-40% B in 35 minutes; A = 10 mM  $\text{NH}_4\text{HCO}_3$ , B =  $\text{CH}_3\text{CN}$ .  $R_t$  about 16.5 minutes.



Pte-Ap

10

*Relative binding affinity.* To determine how well these compounds competes with  $^3\text{H}$ -folic acid for binding to the folate receptor(FR)-positive cell line, KB (available from the American Type Culture Collection, ATCC #CCL-17), a binding assay was conducted. The relative affinity of various folate derivatives was determined according to the method described by Westerhoff et al. (Mol. Pharm., 1995, 48:459-471) with slight modification. Briefly, folate receptor-positive KB cells were gently trypsinized in 0.25% trypsin in phosphate-buffered saline (PBS) at room temperature for 3 minutes and then diluted in folate-free RPMI 1640 media (FFRPMI) (Gibco) supplemented with 10% heat-inactivated fetal calf serum. Following a 5 minute 800 x g spin and one PBS wash, the final cell pellet was suspended in FFRPMI (no serum). Cells were incubated for 15 minutes on ice with 100 nM of  $^3\text{H}$ -folic acid in the absence and presence of increasing concentrations of pteroate-containing test articles. Samples were centrifuged at 10,000 x g for 5 minutes, cell pellets were suspended in buffer, transferred to individual vials containing 5 mL of scintillation cocktail, and then counted for radioactivity. Negative control tubes contained only the  $^3\text{H}$ -folic acid in FFRPMI (no competitor). Positive control

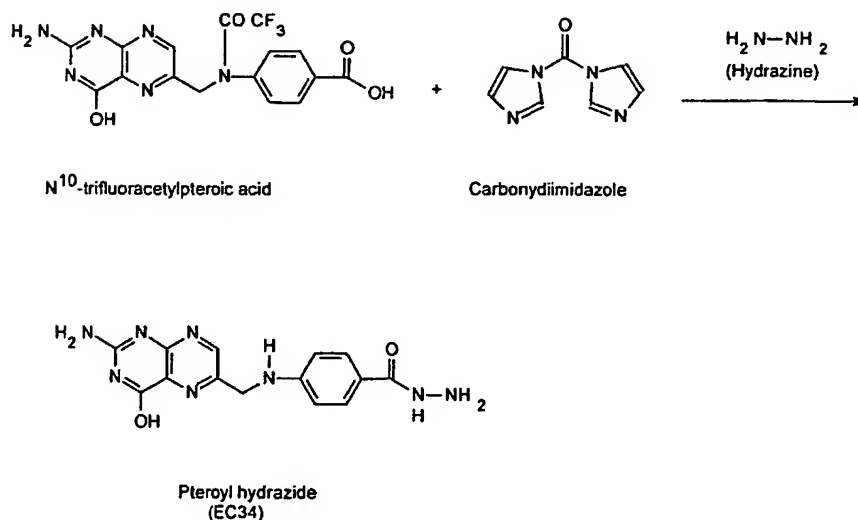
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tubes contained a final concentration of 1 mM folic acid, and counts per minute (CPM) measured in these samples (representing non-specific binding of label) were subtracted from all samples. Relative affinities were defined as the inverse molar ratio of compound required to displace 50% of  $^3\text{H}$ -folic acid bound to folate receptor on KB cells, and the relative affinity of folic acid for the folate receptor was set to 1.

*Results.* The result of the binding assay are shown in Figure 6. The ester ACBP showed a relative binding activity of 0.46 compared to folic acid, and an EC<sub>50</sub> of 204 nM compared to 93.4 nM for folic acid. The folate analog containing an amide bond, Pte-AP, showed a relative binding activity of 0.48 compared to folic acid, and an EC<sub>50</sub> of 193 nM compared to 93.4 nM for folic acid.

#### Example V.

#### Pteroyl Hydrazide and Derivative



*Synthesis of pteroyl hydrazide (Pte-hydrazide).* N<sup>10</sup>-trifluoroacetylpterioic acid (40 mg, 0.098 mmol) and carbonyldiimidazole (25 mg, 0.154 mmol) were dissolved in 2 mL of dimethylformamide and stirred under argon at room temperature for 40 minutes. Hydrazine (40  $\mu$ L; 1.28 mmol) was added to the reaction vessel via syringe. A precipitate immediately formed. Following 15 minutes of stirring, several mL of deionized water were added, and the product was isolated by centrifugation. No further purification was needed.

*Synthesis of pteroylhydrazido-BTCA-DAS.* The synthesis of pteroylhydrazido BTCA-DAS is shown in Figure 7. To a solution of diacetoxyscirpenol (DAS) (50 mg, 0.137 mmol) in 2.5 mL CH<sub>3</sub>CN was added 30 mg (0.137 mmol) benzenetetracarboxylic dianhydride followed by 24  $\mu$ L Hünig's base (17.7 mg, 0.137 mmol, also known as DIPEA, diisopropylethylamine). The reaction mixture was stirred 1.5 hour under argon at room temperature. Some DAS remained, so an additional 6 mg anhydride was added and stirring was continued an additional 1 hour and 10 minutes. Pteroyl hydrazide (59 mg, 0.17 mmol) in 2.5 mL anhydrous dimethylsulfoxide (DMSO) was added, followed by an additional 24  $\mu$ L (0.137 mmol) of Hünig's base. The reaction was stirred for 1 hour and 10 minutes and precipitated by the addition of ethanol.

*Binding activity.* The binding activity of pteroyl hydrazide and pteroylhydrazido-BTCA-DAS was determined using the assay described in Example IV.

*Results.* The results of the binding assay are shown in Figure 8. Pteroyl hydrazide showed a relative binding activity of 0.74 compared to folic acid, and an EC<sub>50</sub> of 94 nM compared to 70 nM for folic acid. Pteroylhydrazido-BTCA-DAS showed a relative binding activity of 0.60 compared to folic acid, and an EC<sub>50</sub> of 116 nM compared to 70 nM for folic acid.

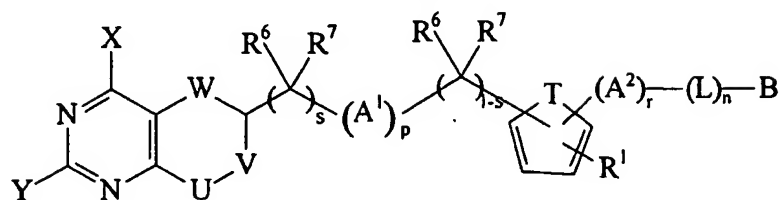
The complete disclosures of all patents, patent applications including provisional patent applications, and publications, and electronically available material (e.g., GenBank amino acid and nucleotide sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and

examples have been provided for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described; many variations will be apparent to one skilled in the art and are intended to be included within the invention defined by

5 the claims.

## WHAT IS CLAIMED IS:

1. A compound having the formula



- 5 wherein

X and Y are each independently selected from the group consisting of halo,  $R^2$ ,  $OR^2$ ,  $SR^3$ , and  $NR^4R^5$ ;

U, V, and W represent divalent moieties each independently selected from the group consisting of  $-(R^6)C=$ ,  $-N=$ ,  $-(R^6)C(R^7)-$ , and  $-N(R^4)-$ ;

- 10 T is selected from the group consisting of S, O, N and  $-C=C-$  such that the ring structure of which T is a member is aromatic;

- $A^1$  and  $A^2$  are each independently selected from the group consisting of  $-C(Z)-$ ,  $-C(Z)O-$ ,  $-OC(Z)-$ ,  $-N(R^4)-$ ,  $-C(Z)-N(R^4)-$ ,  $-N(R^4)-C(Z)-$ ,  $-O-C(Z)-N(R^4)-$ ,  $-N(R^4)-C(Z)-O-$ ,  $-N(R^4)-C(Z)-N(R^5)-$ ,  $-O-$ ,  $-S-$ ,  $-S(O)-$ ,  $-S(O)_2-$ ,  $-N(R^4)S(O)_2-$ ,  $-C(R^6)(R^7)-$ ,  $-N(C\equiv CH)-$ ,  $-N(CH_2-C\equiv CH)-$ ,  $C_1-C_{12}$  alkyl and  $C_1-C_{12}$  alkoxy; where Z is oxygen or sulfur;

$R^1$  is selected from the group consisting of hydrogen, halo,  $C_1-C_{12}$  alkyl, and  $C_1-C_{12}$  alkoxy;

- 20  $R^2$ ,  $R^3$ ,  $R^4$ ,  $R^4$ ,  $R^5$ ,  $R^5$ ,  $R^6$  and  $R^7$  are each independently selected from the group consisting of hydrogen, halo,  $C_1-C_{12}$  alkyl,  $C_1-C_{12}$  alkoxy,  $C_1-C_{12}$  alkanoyl,  $C_1-C_{12}$  alkenyl,  $C_1-C_{12}$  alkynyl,  $(C_1-C_{12}$  alkoxy)carbonyl, and  $(C_1-C_{12}$  alkylamino)carbonyl;

- 25  $R^6$  and  $R^7$  are each independently selected from the group consisting of hydrogen, halo,  $C_1-C_{12}$  alkyl, and  $C_1-C_{12}$  alkoxy; or,  $R^6$  and  $R^7$  are taken together to form  $O=$ ;

$R^6$  and  $R^7$  are each independently selected from the group consisting of hydrogen, halo,  $C_1$ - $C_{12}$  alkyl, and  $C_1$ - $C_{12}$  alkoxy; or,  $R^6$  and  $R^7$  are taken together to form  $O=$ ;

L is a divalent linker;

5 n, p, r and s are each independently either 0 or 1; and

B is hydrogen or a leaving group;

provided that the linker L does not include a naturally occurring amino acid covalently linked to  $A^2$  at its  $\alpha$ -amino group through an amide bond.

10 2. The compound of claim 1 having binding affinity for a folate receptor molecule.

3. The compound of claim 1 wherein

15 X and Y are each independently selected from the group consisting of hydrogen, halo,  $CH_3$ , OH, SH and  $NH_2$ ;

U, V and W represent divalent moieties each independently selected from the group consisting of  $-CH=$  and  $-N=$ ;

20  $A^1$  is selected from the group consisting of  $-C(Z)-$ ,  $-NH-$ ,  $-N(CH_3)-$ ,  $-O-$ ,  $-S-$ ,  $-S(O)-$ ,  $-S(O)_2-$ ,  $-CH_2-$ ,  $-CH(CH_3)-$ ,  $-C(CH_3)_2-$ ,  $-N(CH_2-C \equiv CH)-$  and  $-N(C \equiv CH)-$  where Z is oxygen or sulfur;

$R^1$  is selected from the group consisting of hydrogen, halo and methyl;

$R^6$  and  $R^7$  are each independently selected from the group consisting of hydrogen, halo,  $CH_3$ , OH, SH and  $NH_2$ ; or,  $R^6$  and  $R^7$  are taken together to form  $O=$ ;

25  $A^2$  is selected from the group consisting of  $-C(Z)-$ ,  $-C(Z)O-$ ,  $-OC(Z)-$ ,  $-N(R^4)-$ ,  $-C(Z)-N(R^4)-$ ,  $-N(R^4)-C(Z)-$ ,  $-O-C(Z)-N(R^4)-$ ,  $-N(R^4)-C(Z)-O-$ ,  $-N(R^4)-C(Z)-N(R^5)-$ ,  $-O-$ ,  $-S-$ ,  $-S(O)-$ ,  $-S(O)_2-$ ,  $-N(R^4)S(O)_2-$ ,  $-C(R^6)(R^7)-$ ,  $C_1$ - $C_6$  alkyl;  $C_1$ - $C_6$  alkoxy; where Z is oxygen or sulfur; and p and r are each 1.

30

4. The compound of claim 3 wherein T is  $-C=C-$ .



5. The compound of claim 3 wherein X is OH.

6. The compound of claim 3 having binding affinity for a folate receptor molecule.

5

7. The compound of claim 1 wherein X is OH; Y is NH<sub>2</sub>; U and W are each -N=; V is -CH=; T is -C=C-; A<sup>1</sup> is -NH-; R<sup>1</sup> is hydrogen; A<sup>2</sup> is -C(O)-, -C(O)O- or -C(O)NH- and is *para* to A<sup>1</sup>; R<sup>6</sup> and R<sup>7</sup> are each H; and p, r and s are each 1.

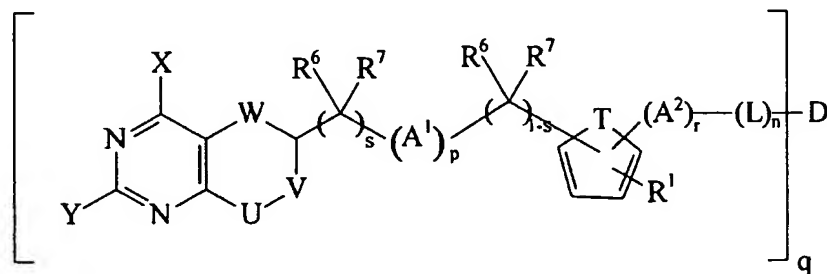
10

8. The compound of claim 7 having binding affinity for a folate receptor molecule.

9. A compound that is isosteric with the compound of claim 7 and that has binding affinity for a folate receptor molecule.

15

10. A ligand-agent conjugate having the formula



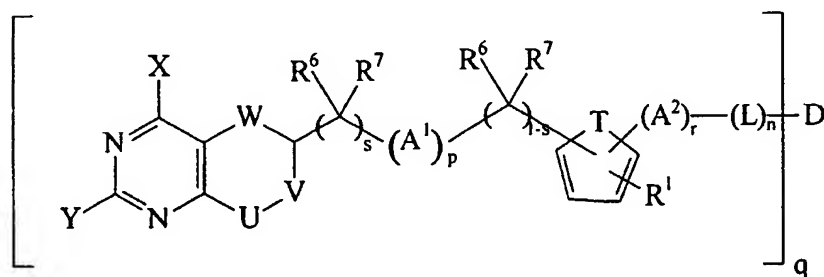
wherein X, Y, U, V, W, T, A<sup>1</sup>, A<sup>2</sup>, R<sup>1</sup>, R<sup>6</sup>, R<sup>7</sup>, L, n, p, r and s are as defined in claim 1;

20

q is an integer  $\geq 1$ ; and,

D is a diagnostic agent or a therapeutic agent.

11. The ligand-agent conjugate of claim 10 that has binding affinity for a folate receptor molecule.
12. The ligand-agent conjugate of claim 10 comprising a metabolically labile linker L.
13. The ligand-agent conjugate of claim 12 wherein the metabolically labile linker L is hydrolytically or reductively cleaved in the cell to release the diagnostic or therapeutic agent Z.
14. The ligand-agent conjugate of claim 12 wherein the metabolically labile linker L comprises a disulfide or an ester.
15. A pharmaceutical composition comprising the ligand agent conjugate of claim 10 and at least one component selected from the group consisting of a pharmaceutically acceptable carrier, excipient, or diluent.
16. A method for delivering a diagnostic agent or a therapeutic agent to a target cell population comprising a folate receptor, the method comprising:  
providing a ligand-agent conjugate having the formula



wherein X, Y, U, V, W, T, A<sup>1</sup>, A<sup>2</sup>, R<sup>1</sup>, R<sup>6</sup>, R<sup>7</sup>, L, n, p, r and s are as defined in claim 1, q is an integer  $\geq 1$ ; and D is a diagnostic agent or a therapeutic agent; and

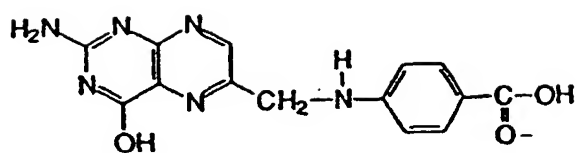
5       contacting the target cell population with an effective amount of the ligand-agent conjugate to permit binding of the ligand-conjugate to the folate receptor.

17. The method of claim 16 wherein D is a diagnostic agent comprising a contrast agent for use in medical imaging.

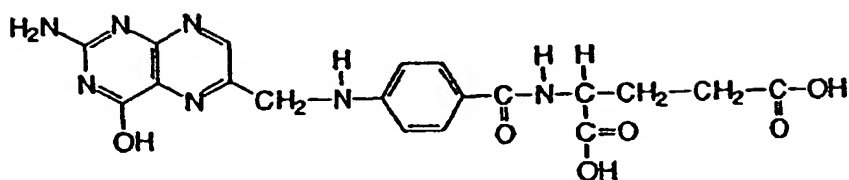
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18. The method of claim 16 wherein the ligand-agent conjugate binds to the cell surface and is not internalized by the cells of the cell population.

19. The method of claim 16 wherein the diagnostic or therapeutic agent D is  
15       internalized by the cells of the cell population.



**Pte (pteroic acid)**



**Pte-Glu (folic acid)**

Fig. 1

# Synthesis of CYK4-013

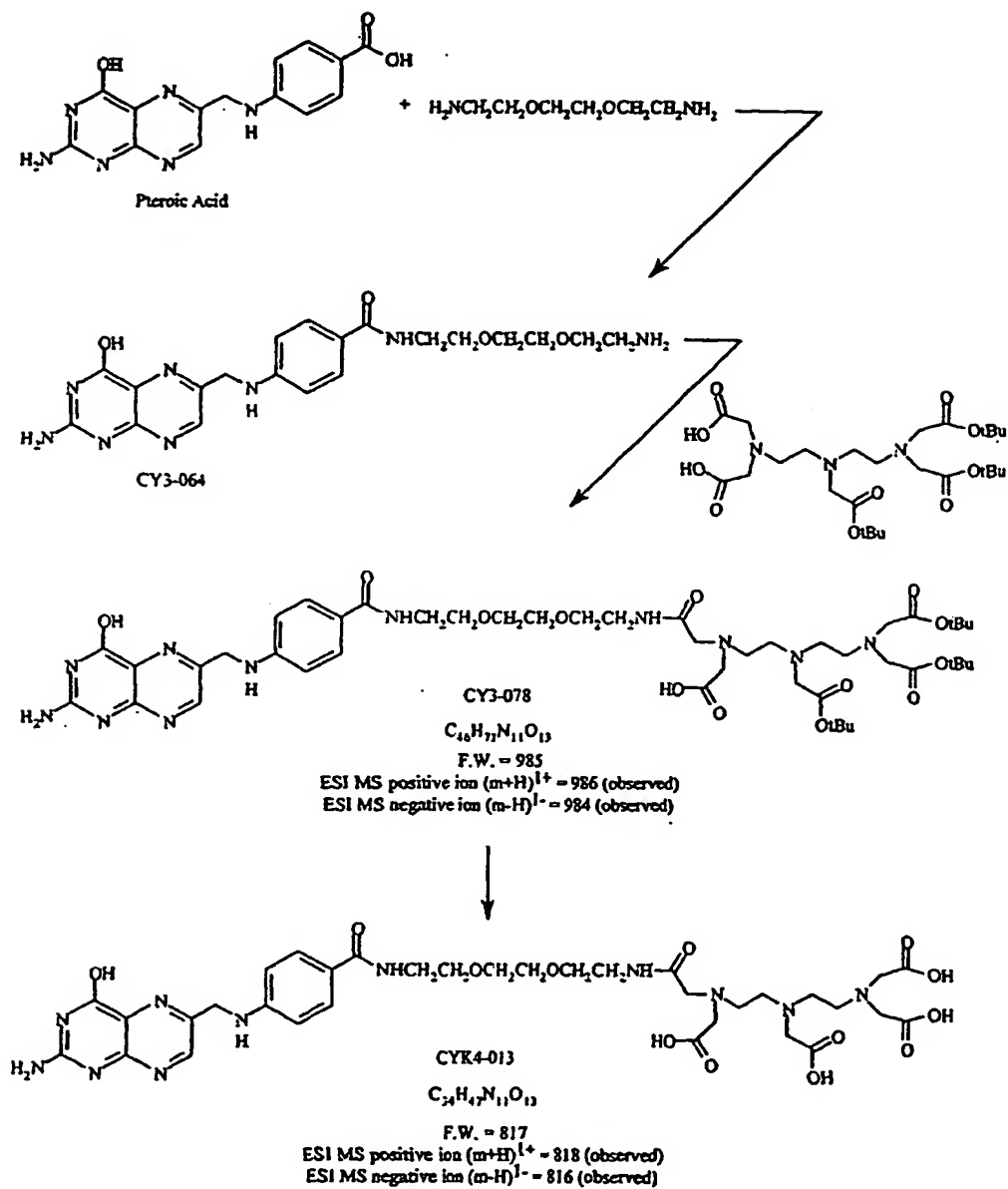
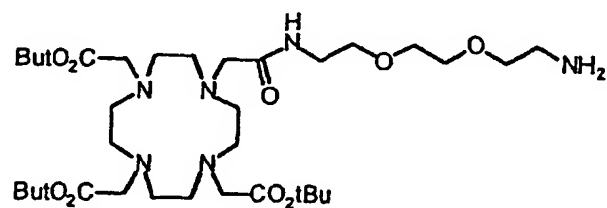
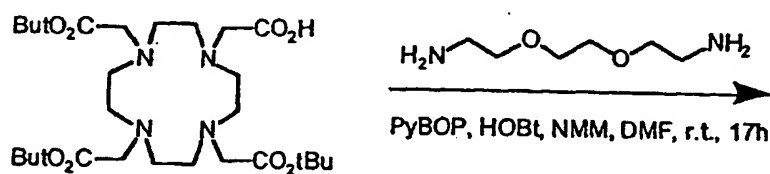
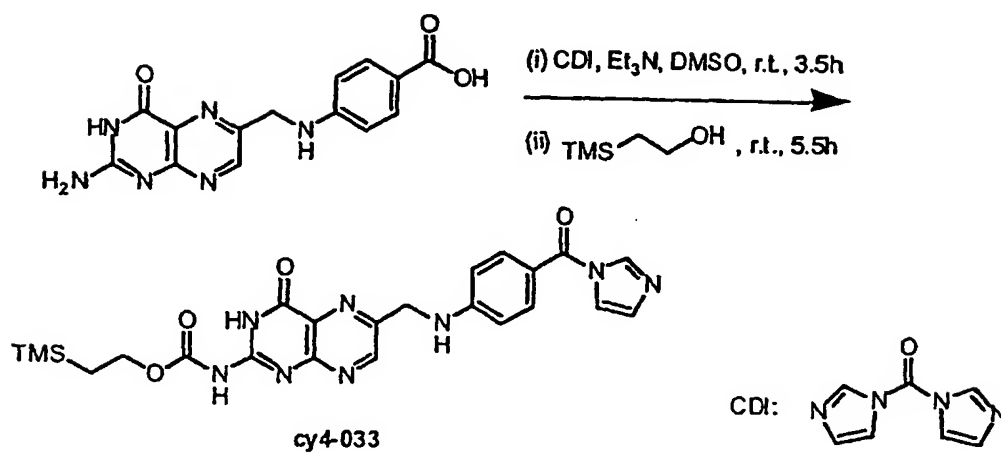


Fig. 2

### Synthesis of cy4-036

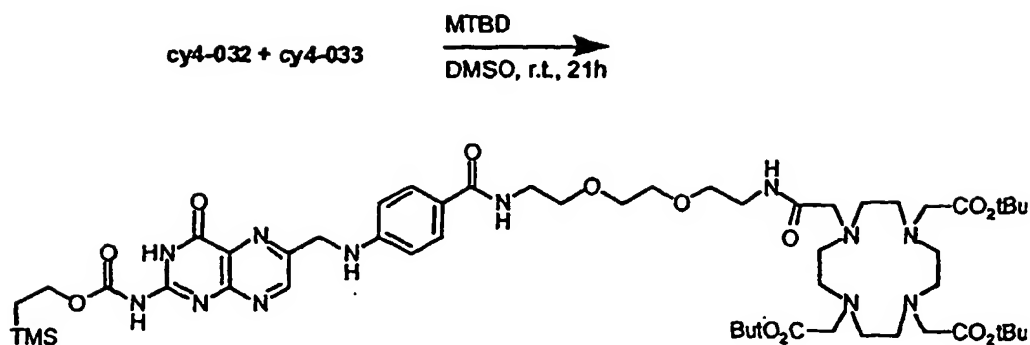


cy4-032

$C_{34}H_{68}N_6O_9 = 702$   
 Electrospray (+): M+H 703

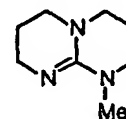
Fig. 3

## Synthesis of cy4-036 (continued)

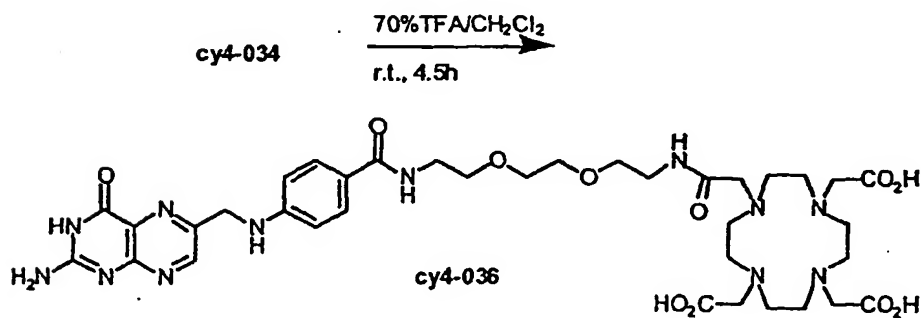


purification by flash column

$C_{54}H_{88}N_{12}O_{13}Si = 1140$   
Electrospray (+): M+H 1141



MTBD



$C_{38}H_{52}N_{12}O_{11} = 828$   
Electrospray (+): 829, 851 Na adduct  
(-): 827, 849

Fig. 3 (cont'd)

Fig. 4.

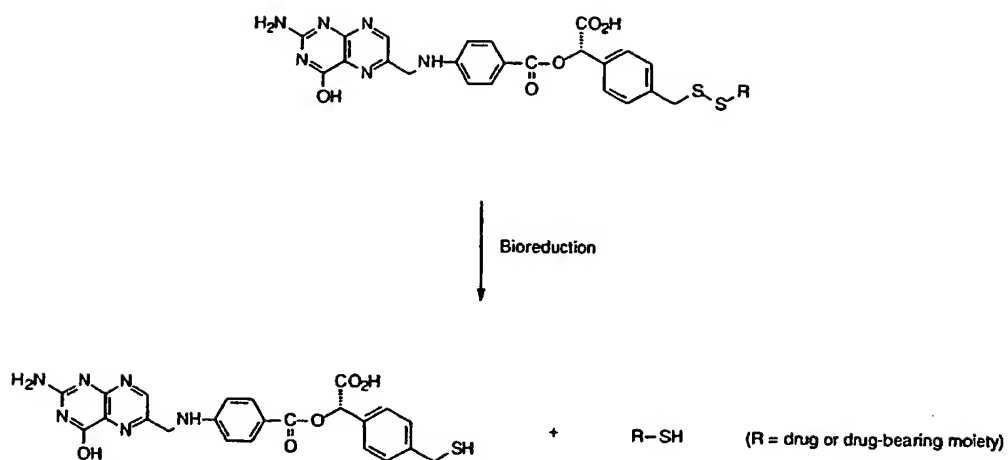
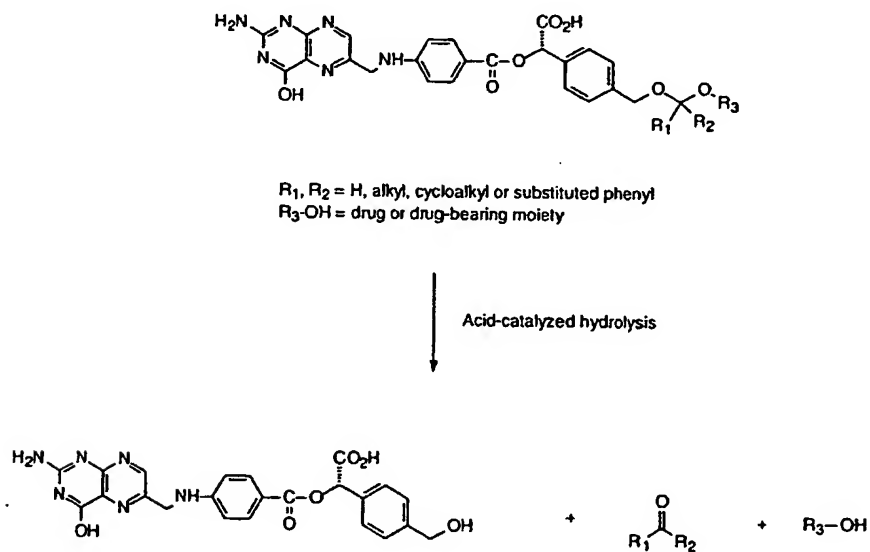


Fig. 5.





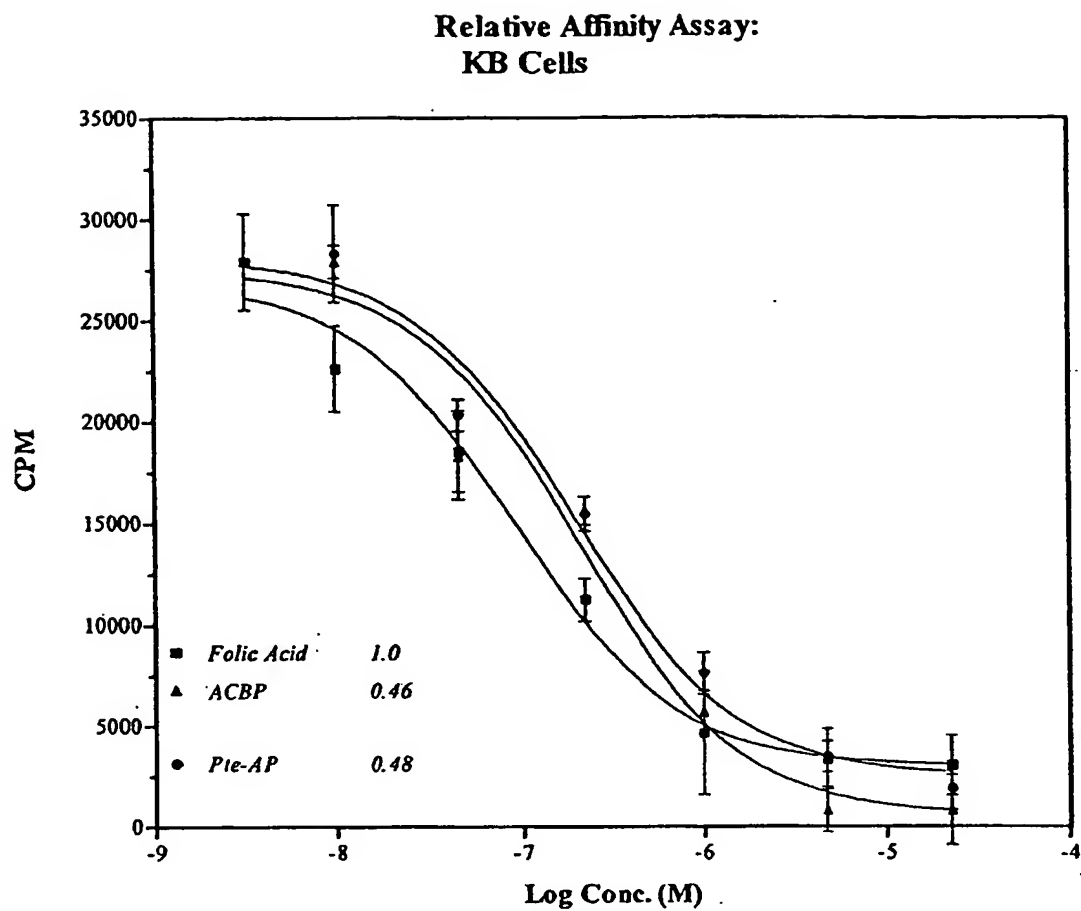


Fig. 6

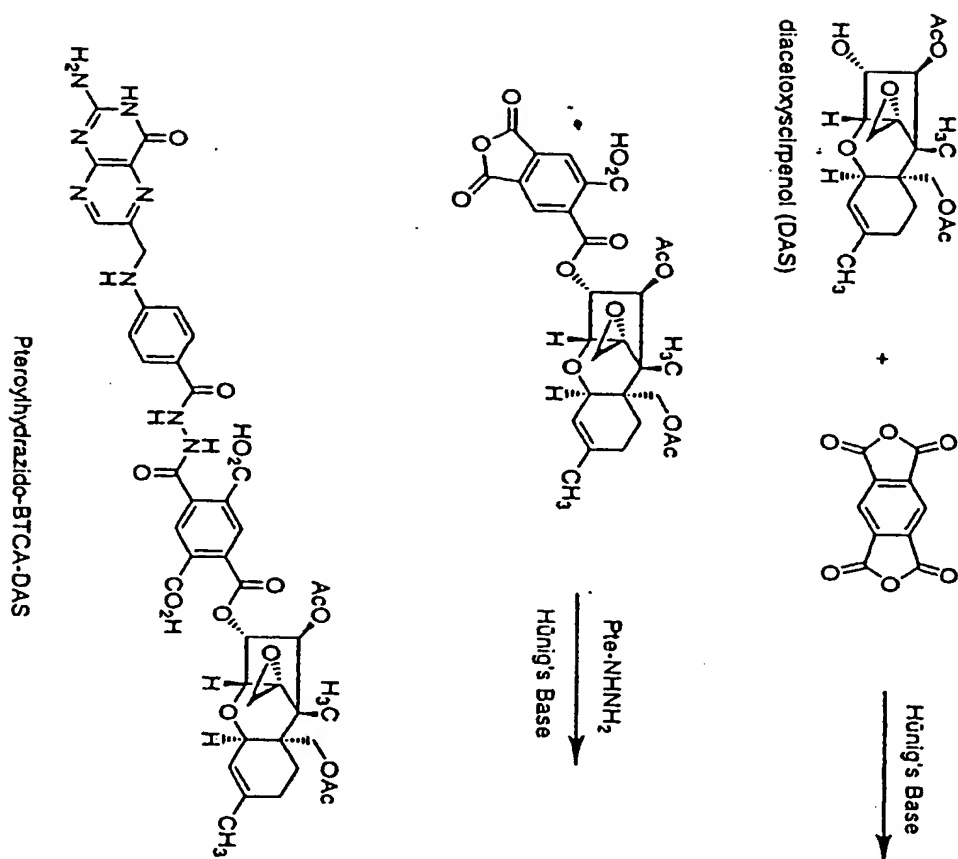


Fig. 7

## Relative Affinity Assay: 15OCT99

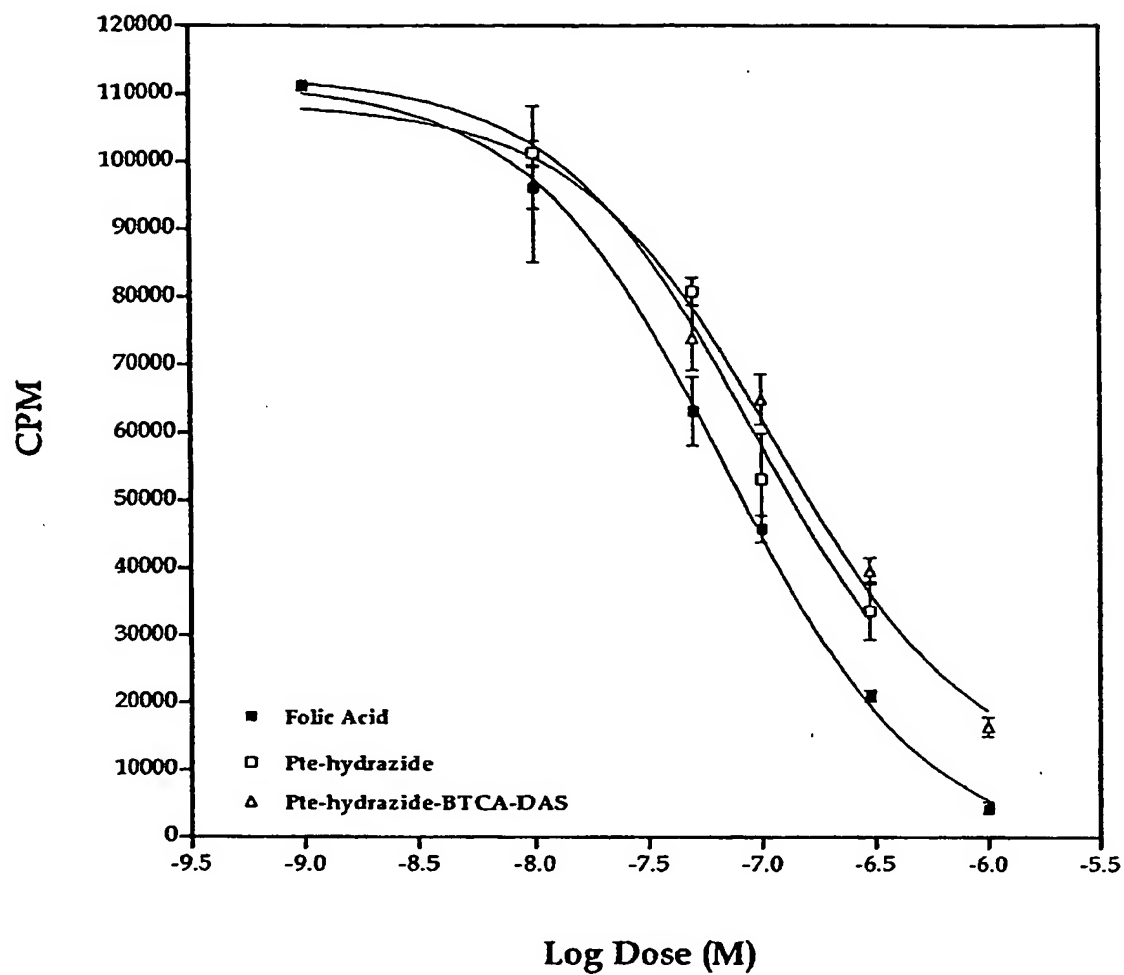


Fig. 8

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/13045

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07D 487/04; A61K 31/4985

US CL : 514/249; 544/257

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
U.S. : 514/249; 544/257

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
CAS ONLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/10651 A1 (MERCK & CO., INC.) 19 March 1998 (19.03.1998), see page 18,	1-9
—	formula (1).	10-19
A		

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

Special categories of cited documents:	
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"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 June 2002 (30.06.2002)

Date of mailing of the international search report

20 SEP 2002

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